

REMARKS

Claim 36 has been added directed to a preferred embodiment previously deleted from claim 2. Support can be found at, e.g., page 7, lines 18-19 in the specification.

Entry of the above amendment is respectfully requested.

Obviousness Rejections

On page 3 of the Office Action, claims 1, 2, 4, 5, 19, 20, 22, 23, 33, and 34 are rejected under 35 U.S.C. 103(a) as being unpatentable over BALOG (Balog, J. M. (2003), Avian and Poultry Biology Reviews, 14(3), pp. 99-126) in view of Emmessar Biotech & Nutrition Ltd. Webpage (hereinafter EMESSAR; available Dec. 4, 2000). On page 6 of the Office Action, claims 21 and 25 are rejected under 35 U.S.C. 103(a) as being unpatentable over Balog and Emmessar as applied to claims 1, 2, 4, 5, 19, 20, 22, 23, 33, and 34, and further in view of COOK (U.S. 6,852,333). On page 7 of the Office Action, claim 24 is rejected under 35 U.S.C. 103(a) as being unpatentable over Balog, Emmessar, and Cook as applied to claims 1, 2, 4, 5, 19, 20, 22, 23, 33, and 34, and further in view of MOLLY (WO 03/043440). On page 9 of the Office Action, claims 1-6, 26-31, 33, and 35 are rejected under 35 U.S.C. 103(a) as being unpatentable over COOK (U.S. 6,852,333) in view of ZHANG (U.S. 6,875,890). On page 11 of the Office Action, claims 7 and 32 are rejected under 35 U.S.C. 103(a) as being unpatentable over Cook in view of Zhang as applied to claims 1-6, 26-31, 33, and 35, and further in view of MOLLY (WO 03/043440).

Applicants respectfully submit that the present invention is not obvious over the cited art combinations, and request that the Examiner reconsider and withdraw these rejections in view of the following remarks.

Initially, Applicants note that in claim 1, they have claimed a method wherein the glycine compound, such as DMG, is administered to poultry either for the purpose of reducing the conversion rate of the feed (FCR) used to raise the poultry or for reducing the incidence of ascites. Claim 19 is specifically directed to the method for reducing the incidence of ascites in poultry, while claim 26 is specifically directed to the method for reducing the FCR.

On pages 3 to 5 the Examiner explains why the use of the glycine compound would be obvious for reducing the incidence of ascites in poultry. His arguments as to why the use of the glycine compound would be obvious for reducing the feed conversion rate (FCR) are given on pages 9 to 11.

According to the Examiner, the use of the glycine compound DMG for reducing the incidence of ascites would be obvious over Balog in view of Emmessar. The invention would more particularly be obvious because Balog teaches that inadequate oxygen (i.e., hypoxia) is the primary cause of ascites and since Emmessar teaches that DMG is used as a poultry feed supplement to enhance oxygen absorption and delivery.

As can be seen on the homepage of Emmessar Biotech & Nutrition Ltd., Emmassar is an Indian company which does not do basic research but which only sells, apart from homeopathy products, a few other nutrient additives, in particular DMG, MSM and TMG. For these products, they mention as much as possible different beneficial effects without referring even to the various articles wherein these effects are mentioned. It is thus very doubtful whether these effects have been effectively proved, and this in a statistically significant way. In this respect, Applicants refer to a brochure of Davinci[®] Laboratories of Vermont relating to the DMG which they also put on the market (see the attached copy of this document, or see the webpage [9](http://www.seekinghealth.com/product_files/1161668603Gluconic%20DMG%20(DMG-</p></div><div data-bbox=)

TB).pdf). For each of the suggested applications of DMG they specifically indicate at the bottom of each page: *“*This statement has not been evaluated by the Food and Drug Administration. This product is not intended to diagnose, treat, cure or prevent any disease”*, indicating that it has not yet been sufficiently proved that DMG has actually all of these effects.

Moreover, based on the teachings of Balog and Emmessar, a skilled person would not try to use DMG for preventing ascites as he would not expect that a DMG administration to poultry would be able to reduce the incidence of ascites. On the contrary, a skilled person would even expect that a DMG administration might increase the risk on ascites. It is indeed known that ascites is basically caused by a hypoxemic condition (see Balog) and that DMG is able to decrease hypoxia to various tissues (see, for example, the next-to-last line on page 10 of the article of Roger V. Kendall and John W. Lawson, “Recent Findings on N, N-Dimethylglycine (DMG): A Nutrient for the New Millenium” (2000), cited on p. 5, l. 4 – 10 of the present application and submitted to the Examiner with the IDS filed on September 20, 2006). However, from the schematic representation of the progression of ascites (pulmonary hypertension) syndrome in broilers in Figure 1 on p. 103 of Balog, it appears that the hypoxemic state, which is at the origin of ascites, is initially compensated through an increased synthesis of erythropoietin, i.e., an increase in hematocrit level, which eventually leads to an elevated blood viscosity that on its turn gives rise to pulmonary hypertension followed by right heart failure (see also p. 2, l. 3 – 16 of the present application). When the prior art teaches that DMG helps in absorption, assimilation, transport and delivery of oxygen to all the individual cells, a skilled person would expect that DMG administration to poultry would also increase the hematocrit level of the blood in order that this blood would be able to transport more oxygen to the different tissues (see the tests results set forth in the present application and in particular the conclusion on p. 17, l. 12 –

14 that DMG supplementation leads indeed to an increase, albeit relatively small, of the hematocrit level). He would further expect that the hematocrit level, which is not only increased by the hypoxemic conditions but which is expected to be increased moreover by the DMG supplementation, would thus lead to a greater and/or faster increase of the blood viscosity and hence to an increased risk on the development of ascites. Consequently, based on the teachings of the prior art, a skilled person would not expect at all that a DMG supplementation might reduce the incidence of ascites in poultry.

The present inventors have found quite by surprise that DMG supplementation does reduce the incidence of ascites in poultry, notwithstanding the fact that it does not lower the hematocrit level in the blood. They found out that the effect of DMG of ascites is not due at all to its described ability to decrease hypoxia but instead to its effect on the NEFA level (Non Esterified Fatty Acid). As described in Bottje and Wideman, 1995 and in Diaz-Cruz et al., 2003 (copies attached), an increase in mitochondrial reactive oxygen species, as a result of an increase in metabolic rate, indeed causes lipid peroxidation mediated damage to the pulmonary vasculature through which oxygenation is deteriorated and hypoxemia aggravated, thus also increasing the risk of the development of ascites. The tests described in the present application have demonstrated that the NEFA level in the blood could be significantly lowered by the DMG supplementation (see p. 16, l. 26 – p. 17, l. 8 of the present application). As disclosed in Avagaro et al., 2003 and in Sarafidis and Bakris, 2006 (copies attached), NEFA induces important vascular effects and dysfunctions, resulting in an increase in arterial pressure. Moreover, endothelial dysfunction of the extrapulmonary conduit arteries results in hypoxia, which also triggers progression towards pulmonary hypertension (Zoer et al., 2009, copy

attached). Hence, the protective mechanism of dietary DMG on development of pulmonary hypertension can, at least partly, be attributed to its effect on fat metabolism.

As to claims 1 and 26, the Examiner considers the use of DMG for reducing the FCR of poultry to be obvious over Cook in view of Zhang.

Cook discloses the use of an antistress agent to reduce the FCR in poultry. The “antistress agent” is defined in Cook as being a compound or composition effective in reducing stress (see c. 7, l. 57 – 58). Agents which simply act as nutritional modifiers are not included in the term “antistress agent” so that the antistress agents used in the method of Cook are not simply nutritional modifiers but have to be also physiological and/or psychological stress reducers per se (see c. 7, l. 60 – 65). This limited definition of the term “antistress agent” was given in Cook to distinguish his method from the prior art, more particularly from the nutritional supplements, disclosed for example in US 5505968 and in US 4600586 for use in reducing the effects of stress on animals (see c. 2, l. 5 – 13). As explicitly mentioned in c. 2, l. 12 – 13, the prior art compositions disclosed in those patents are no antistress agents since they do not treat stress per se.

Zhang discloses in c. 1, l. 44 – 48 the use of DMG as a nutritional supplement to improve physical and mental performance by helping the body adapt to the various forms of stress. DMG is thus used in Zhang for reducing the effects of stress on humans or animals, and not to treat stress per se, so that it is not an “antistress agent” as defined by Cook. Consequently, a skilled person would not use DMG as “antistress agent” in the method of Cook and would thus not arrive at the method for reducing the FCR as defined in claims 1 and 26.

Applicants have noted that at the end of the paragraph in c. 1, l. 44 – 55, Zhang mentions that DMG is an anti-stress nutrient, without describing what is to be understood by the term

“anti-stress nutrient” and without providing, just like Emmessar, any further experimental evidence therefor. Effects of DMG have thus also been taken over by Zhang from some earlier publications. From the more scientific publication of Kendall, it appears however that although DMG has been described in relation to stress, it is not an “antistress agent” as defined by Cook. Kendall describes for example (in the last paragraph of p. 3 and the first paragraph of p. 4) that DMG can strengthen the immune system and that a weakened immunity is associated with degenerative conditions, aging and increased stress. When the immune system of the poultry is weakened, DMG may thus strengthen this immune system and may in this way avoid stress. DMG itself has, however, no effect on the stress itself and is thus not an antistress agent as intended by Cook since DMG does not treat stress per se. A skilled person would thus again not use the DMG disclosed as being an “anti-stress nutrient” in Zhang as an “antistress agent” in the method of Cook. The method for reducing the FCR as defined in claims 1 and 26 is thus not obvious over Cook in view of Zhang.

Accordingly, Applicants submit that the present invention is not obvious over the cited art combinations, and withdrawal of these rejections is respectfully requested.

Conclusion

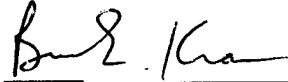
In view of the above, reconsideration and allowance of this application are now believed to be in order, and such actions are hereby solicited. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

AMENDMENT UNDER 37 C.F.R. § 1.111
Application No.: 10/599,119

Attorney Docket No.: Q96506

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

Respectfully submitted,



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WASHINGTON OFFICE

23373

CUSTOMER NUMBER

Date: January 25, 2010

THE DISCOVERY COMPANY[™]

GLUCONIC[®] DMG

GLUCONIC[®] DMG

Gluconic[®] DMG is DaVinci's Patented and Branded N,N-Dimethylglycine (DMG) a dietary supplement to support immune, detoxification, and circulatory functions.* It also supports physical and mental performance and cardiovascular function.*

WHAT IS IT?

DMG is the simplest amino acid, glycine, where two hydrogen atoms have been replaced with methyl (CH₃) groups on its nitrogen atom. Research shows it to be physiologically active and important to cell metabolism. DMG supplies essential methyl groups for modification, building, and detoxifying many components in the body.

WHERE IS IT FOUND?

DMG is a natural substance found in low levels in the body and in certain foods like meat (liver), beans, seeds, and grains. DMG is not found in high levels in your body because it is a water-soluble intermediary metabolite, meaning it has a temporary role in the cell cycle before it is rapidly broken down into other substances that your body needs. The human body can produce small amounts of DMG from choline and betaine, but supplementation can provide increased levels of DMG resulting in many health benefits to the body.

WHAT ARE THE BENEFITS OF SUPPLEMENTATION?

DMG can enable a person to function at their optimal mental and physical level.* In fact, in the best-selling book *Prescription for Nutritional Healing* DMG is recommended for over 48 different ailments.

DMG SUPPORTS:

- Neurological function*
- Physical and mental performance.*
 - energy levels.*
 - reduced lactic acid levels.*
 - shorten recovery time after strenuous exercise.*
 - oxygen utilization.*
- Stress management.*
- Detoxification and liver function*
- Cardiovascular functions*
 - circulation and vascular health.*
 - blood pressure, cholesterol, triglycerides, and homocysteine levels within normal ranges.*
- Immune response*
 - strengthening of both arms of the immune system.*
 - antibody, lymphocyte and cytokine production.*
 - anti-viral, anti-bacterial, and anti-tumor properties and modulates inflammation responses.*

- Verbal communication & social interactions in autistic individuals.*
- Glucose metabolism (storage and utilization).*
- Overall body health by acting as an indirect methyl donor and supporting the biosynthesis of vitamins, hormones, neurotransmitters, antibodies, and nucleic acids and other metabolically active molecules.*

HOW DOES IT WORK?

DMG's ability to supply one and two carbon molecules to the cell, as well as its ability to contribute to the formation of S-Adenosylmethionine (SAM-e) and support methylation reactions, may help explain DMG's broad metabolic activity and therapeutic effect on the body.

DMG provides building blocks for the biosynthesis of many important substances such as methionine, choline, several important hormones, neurotransmitters, and DNA which support mental activity, high energy levels, a strong immune system, improved oxygen utilization, and improved functioning of many important organs.*

DMG supports methylation production, a biochemical process that is essential to life, health, and regeneration of body cells.* Transmethylation is a reaction where a methyl group is transferred from one molecule to another. Vitamins, hormones, neurotransmitters, enzymes, nucleic acids, and antibodies depend on the transfer of methyl groups to complete their synthesis. By its ability to donate its methyl groups, DMG supports production of SAM-e, the most active transmethylation agent in the body.* SAM-e is involved in over 41 different transmethylation reactions in the body.

The one carbon units produced from DMG can be used to form the essential amino acid Methionine from Homocysteine. This transformation requires folic acid, NAD⁺, FAD and Vitamin B₁₂. Methionine in turn is used to produce SAM-e, the active transmethylation agent in the body. This process keeps the cells of the body in a high state of transmethylation potential. It also reduces the concentration of Homocysteine in the blood, which tends to build up when there is a low availability of methyl groups. High levels of Homocysteine have been shown to cause arteriosclerosis. DMG may play an important role in keeping Homocysteine at a safe level.

DMG also generates two carbon molecules such as Glycine, Serine and the ethanolamines, all of which are beneficial to the life of the cell. For example, Glycine functions as an important inhibitory neurotransmitter of the central nervous system and is used to produce phosphocreatine, a high energy phosphate molecule used in muscle tissue and in the tissue of the central nervous system.

ATHLETIC PERFORMANCE*

Athletes use DMG to support overall performance and endurance, to benefit oxygen utilization, to reduce lactic acid build-up, and to reduce recovery time of muscles after strenuous exercise.* DMG's apparent energy enhancing (ergogenic) effects could be due better cellular respiration and enhanced carbohydrate and lipid metabolism.*

The evidence that DMG can enhance the performance of athletes is quite strong. The research, along with actual field evaluation, shows that DMG is beneficial to endurance athletes (runners, team sports) as well as short-timed events (weight lifters, sprinters). Research shows that DMG is not only beneficial for overall performance and recovery, but it will also support the immune defense of the individual athlete and make them less susceptible to infections or other illnesses.

IMMUNE RESPONSE SUPPORT*

It supports health through immune system modulation and antioxidant protection.* DaVinci holds four U.S. Patents on DMG based on over 20 years of extensive research into its role in the immune response.

A strong immune system is an important part of staying healthy and avoiding serious illness. DMG is a nutritional factor that can strengthen the immune system and bring about a more effective response against any disease causing antigens or pathogens.*

Research shows that DMG significantly stimulates B-cells to produce higher antibody responses (humoral branch) and potentiates a greater activity of T-cells and macrophages (cellular immunity branch). DMG therefore enhances both arms of the immune response.

1. DMG invoked a humoral or antibody response in rabbits given typhoid vaccine demonstrating B-cell activity.
2. DMG increased lymphocyte population in human.
3. A double-blind study involving 20 human subjects showed DMG to be effective in stimulating both a humoral (antibody) as well as a cellular-mediated immune. A 4.5-fold increase in antibody titer was seen in the test group as compared to the control group.

As a result of this work FoodScience Corporation was granted patent U.S. Patent #4,385,068 in may of 1982 and #4,631,189 in December of 1986 on DMG and its role in potentiating the immune response.*

The foodscience Corporation was granted U.S. Patent #5,118,618 in June of 1992 on DMG and enhancement of antibody production.

In a study done by the U.S. Army by Dr. Ivin at the Medical Institute of Infectious Diseases at Ft. Detrick, Maryland evaluated the effects of DMG with guinea pigs given an anthrax vaccine. No significant increase in antibody titers were seen in the DMG fed animals as compared to the vaccinated controls. However, when the animals were subsequently challenged with a potentially lethal dose of virulent anthrax bacilli, 50% of the control animals given the vaccine died. Among the vaccinated animals given the vaccine and DMG, not one of the DMG fed animals died. These remarkable findings seem to indicate that although no increase in antibody titers were seen in the DMG fed animals as compared to the controls, the DMG fed animals demonstrated an enhanced immunity which must have been due to an enhanced cellular response (T-cells, B-cells).

Due to the ability of DMG to regulate cytokines (cell messengers) it seems to help improve cell recognition and communication, which helps make cellular processes more efficient. It helps regulate the immune system as well as normal inflammatory responses.

This effect was found in a rat study done at Clemson University by Dr. John Lawson. DMG was tested on a Rheumatoid Collagen II induced model used in the rat. Under these conditions, 70% of the control rats developed arthritis, but only 30% of the test rats given DMG developed arthritis. This study shows that DMG, probably through modulation of the immune system, may be helpful in conditions where immune system regulation or inflammation is a major symptom.

FoodScience Corporation was granted U.S. Patent #5,026,728 in June of 1991 on the use of DMG to treat arthritis and inflammation based on this research.

Aside from prolonged cell life in healthy cells it also supports apoptosis or program cell death in cells that may be damaged or infected.

Work done by Dr. Lawson at Clemson University in a mouse study using a B-16 melanoma model showed that mice receiving DMG had a significantly higher antibody count against the B-16 antigen and the growth of infected cells was significantly retarded as compared to the controls. The DMG

mice lived longer than the controls and had fewer infected cells. DMG also seemed to keep the infected cells together so they could not spread. At the end of the study, all of the control mice had died but of those mice receiving DMG, 71% were still surviving. In a CAM (chorioallantoic membrane) chick embryos assay DMG reduced infected cell growth by 60% as compared to controls. It is now known that DMG increases the production of Tumor Necrosis Factor-alpha.

These studies indicate that DMG may give important nutritional support to the immune system. As a result of this work FoodScience Corporation was granted U.S. patent # 4,994,492 in February of 1991 for DMG's role in treating melanoma.

STRESS MANAGEMENT*

Gluconic® DMG is an adaptogen that can be used to maintain good health and benefit performance and productivity.* As a metabolic enhancer, DMG supports the body in times of stress, throughout the aging process, and during immune system challenges.*

CARDIOVASCULAR SUPPORT*

DMG supplementation is very beneficial to circulation and cardiovascular function.* It supports oxygen delivery to the heart and improves several characteristics of the blood including healthy cholesterol and triglyceride levels.*

A four-year clinical evaluation was done by Mitchell Pries, M.D. of Palmdale, CA, where DMG was administered to over 400 cardiovascular patients over a four-year period. Dr. Pries reported major improvements in the following areas:

1. Increased feeling of well-being.
2. Improvement in circulatory insufficiency.
3. Decrease in elevated cholesterol and triglycerides.
4. Reduction in angina pain.
5. Fewer arrhythmias.
6. Decrease of high blood pressure.
7. Improvement in heart response to stress tests.

Most of the patients in the study had cholesterol levels of greater than 250+ mg/dl. After taking 250 mg of DMG for 3 months, most showed a drop in blood cholesterol to approximately 200 mg/dl. A major drop was seen for the triglycerides as well. The patients underwent standard diagnostic testing including blood chemistries, electrocardiograms, and Doplar blood vascular readings. Dr. Pries concluded that 125 mg of DMG, taken twice daily, was effective in producing major positive responses in his cardiovascular patients.

NEUROLOGICAL SUPPORT*

A lack or imbalance of neurotransmitters can cause a whole series of brain and neurological dysfunctions. DMG can act as a precursor to a number of amino alcohols and acids that aid brain function. In several reports, case studies and research papers DMG was shown to reduce frequency and

severity of seizures.* It may be that DMG is able to cross the blood brain barrier better than Glycine, thereby acting as a source of the Glycine neurotransmitter in the brain. Several other explanations are also possible.

AUTISM*

There is a growing body of evidence that DMG is beneficial to individuals with autism.* Autism is a biological brain disorder of unknown causes that results in a wide range of puzzling and disturbing social and personal behavior patterns. DMG may modify and improve the behavior, social interaction, verbal communication, and disturbing activities of autistic children.*

Two studies have demonstrated that DMG can modify and improve the behavior, social interaction, verbal communication and disturbing activities of autistic children. Significant improvements were seen. DMG showed statistically significant improvement on all five ABC scales used to evaluate effectiveness: Irritability, Lethargy, Stereotypy, Hyperactivity and Inappropriate Speech. The Placebo group showed improvement only on the lethargy scale.

Since 1991, Dr. Bernard Rimland from the Autism Research Institute has recommended that parents give DMG to their autistic children.

These results confirm the hundreds of communications that the Autism Research Institute, in San Diego, CA, under the direction of Dr. Bernard Rimland, have received from excited parents who have seen remarkable changes in their autistic children after using DMG. Areas where parents noted improvement include:

- Better Verbal Communication
- Better Eye Contact
- Improved Loving Attitude
- Better Social Interaction

The improvement seen on DMG may be due to improved methylation, better oxygen utilization, reduction of lactic acid formation and a possible decrease in potential seizure activity. Perhaps the most relevant research on DMG relative to autism may be in the immunological area. The immune system may be abnormal in people with autism. These include decreased number of helper T-cells and B-cells, reduced natural killer cell activity, inhibition of macrophage activity and increased interferon levels. DMG's ability to modulate the immune system may work to correct a basic defect in the immune system of autistic children that is

responsible for the symptoms being displayed. Further studies in this area are needed.

CARBOHYDRATE METABOLISM*

There is good evidence that DMG may enhance carbohydrate metabolism. There have been many individual reports from physicians who have attested to DMG's value in lowering blood sugar levels in diabetic patients. Problems resulting from impaired circulation, including gangrene, pain in the lower extremities, wounds and ulcers on the legs and feet, all responded well to DMG supplementation.

DMG may be beneficial for hypoglycemics because it assists in reducing elevated lactic acid levels and supplies a rich source of methyl groups for transmethylation reactions in the body.

DETOXIFICATION & LIVER SUPPORT*

DMG supports the production of intracellular glutathione which is the master antioxidant of all cells and is always in great demand by the liver to support liver detoxification processes.*

DMG is beneficial in support of liver metabolism and detoxification. By improving oxygen utilization, DMG can help eliminate hypoxia to the tissues and aid in the elimination of toxins from the blood stream.

Patients undergoing either detoxification would benefit from DMG supplementation to support detoxification, oxygen uptake and circulation to the extremities of the body, and cellular metabolism for increased energy levels. Patients receive a boost in their sense of well being and energy levels which may relate to improved blood glucose levels.

CONCLUSION:

This Technical Bulletin has only touched the surface of the many areas where DMG has been found to be beneficial including the immune response, detoxification, mental and physical performance, enhancement to cellular metabolism and many others.*

SAFETY:

DMG can be safely combined with any nutritional or therapeutic product without negative side effects. Gluconic® DMG is very effectively absorbed from the digestive tract and mouth. DMG is a water soluble nutrient and the enzyme system in the body effectively converts the substance into metabolites that are either used by the body or are safely excreted from the body. DMG is a safe substance that has been recommended by thousands of clinicians and doctors for almost three decades without adverse or negative side effects.*

DMG has been found to be an extremely safe food substance as demonstrated by a series of animal studies conducted at the Medical University of Southern California. Meduski has

reported that DMG HCl has an LD₅₀ (lethal dose to 50% of the animal population) of 7,400 mg per kg of animal body weight in the rat. This amount is generally regarded as nontoxic. Feeding studies at reasonably high levels have demonstrated DMG's long term safety. A two year feeding study in rats produced no health problems, even when fed at a level 1/10 the LD₅₀ on a daily basis (740 mg/kg of body weight per day).

SUGGESTED RANGES OF USE FOR DMG:

Taken Daily in Divided Doses

- From *Building Wellness with DMG*-

General Use & Anti-Aging*	125-500 mg
Sports Practice and Fitness*	375-1000 mg
Endurance Sports*	1000-2500 mg
Immune Response (Prevention)*	375-750 mg
Compromised Immune System*	750-1200 mg
Cardiovascular & Circulatory*	375-1000 mg
Diabetics and Hypoglycemics*	375-1000 mg
Autism, ADD, & Seizures*	375-1000 mg
Cancer*	1500-2500 mg
Autoimmune diseases (Lupus)*	750-1000 mg
Chronic Fatigue/Fibromyalgia*	1000-1500 mg
Liver Detoxification*	750-1000 mg
Respiratory (Asthma/Allergies)*	750-1000 mg
Stress*	250-750 mg

FORMULAS:

Gluconic® DMG 125 mg- Sublingual

Active Ingredient:

N,N-Dimethylglycine 125 mg

Other ingredients: none.

Gluconic® DMG 125 mg Chewable

Active Ingredient:

N,N-Dimethylglycine 125 mg

Other ingredients: xylitol, malic acid, silicon dioxide, vegetable stearate.

Gluconic® DMG 250 mg Chewable

Active Ingredient:

N,N-Dimethylglycine 250 mg

Other ingredients: xylitol, malic acid, silicon dioxide, vegetable stearate.

Gluconic® DMG 300 mg Liquid

Active Ingredient:

N,N-Dimethylglycine 300 mg

Other ingredients: Purified water.

Gluconic® DMG 500 mg Chewable

Active Ingredient:

N,N-Dimethylglycine 500 mg

Other ingredients: xylitol, malic acid, natural cherry flavor, zeaxanthin, vegetable stearate.

RECOMMENDATIONS:

Suggested Use (125 mg pure tablet): Dissolve 1 tablet in mouth, 1 to 6 times daily.

Suggested Use (125 mg chewable tablet): Take 1 chewable tablet, 1 to 6 times daily.

Suggested Use (250 mg): Take 1 chewable tablet, 1 to 3 times daily.

Suggested Use (500 mg): Take 1 chewable tablet, once or twice daily.

Suggested Use (300 mg Liquid DMG): The following is recommended:

Age: Maintenance: Active:

Under

2 yrs 3-5 drops daily 5 drops twice daily

2-5 yrs 5 drops daily 0.5 ml, twice daily

5-12 yrs 0.5 ml daily 1.0 ml twice daily

12 yrs-

adult 1.0 ml daily 1.0 ml, 2-4 times daily

NOTE: Our water is purified through Reverse Osmosis with a 2-micron filter.

1 drop = 15 mg DMG

PRODUCT FEATURES

We offer 4 potencies (125, 250, 300, 500 mg) and 3 delivery systems (sublingual, chewable, liquid).

DMG is a multi-patented product! Based on 20 years of extensive research into the benefits of DMG on Immune System Functions DMG was awarded four U.S. patents.*

DMG chewable tablets are sweetened with xylitol.

Xylitol

- All Natural Sweetener (sugar substitute) 40% less Calories
- Found naturally in birch trees, fruits, & vegetables
- Our bodies produce a few grams of Xylitol daily
- FDA approved as dietary food supplement since 1963
- FDA approved as a nutritional sweetener
- 30 years of use and research in Scandinavia

ORDERING INFORMATION:

Product Name: Gluconic® DMG

Form: Available as a sublingual or chewable tablet as well as a liquid. Available in a 125 mg, 250 mg, 300 mg or 500 mg strength.

Gluconic® DMG 300 mg Liquid
2592.2 (2 fl oz/60 ml Liquid)

Gluconic® DMG 125 mg Sublingual Tablets

2380.3 (30 Tablets)

2380.6 (60 Tablets)

2380.9 (90 Tablets)

Gluconic® DMG 125 mg Chewable Tablets
2387.12 (120 Tablets)

Gluconic® DMG 250 mg Chewable Tablets
2382.6 (60 Tablets)
2382.9 (90 Tablets)

Gluconic® DMG 500 mg Chewable Tablets
2393.6 (60 Cherry Flavored Tablets)

Sold exclusively through health care practitioners.

ORDER BY:

Phone: 1-800-325-1776

Website: www.davincilabs.com

E-Mail: info@davincilabs.com

Fax: 1-802-878-0549

REFERENCES:

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Potential Role of Free Radicals in the Pathogenesis of Pulmonary Hypertension Syndrome

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1 INTRODUCTION

The generation of free radicals and reactive oxygen species (ROS) have been associated with several health problems including cancer, diabetes, atherosclerosis, cystic fibrosis, kwashiorkor, HIV infection, altitude sickness and many others¹⁻⁹. Consequently, there has been considerable research effort in the medical community devoted to free radical biochemistry. It now appears that there may be an involvement of free radicals and antioxidant mechanisms in the pathogenesis of ascites or pulmonary hypertension syndrome (PHS)^{10,11}, a costly worldwide problem in the poultry industry. The intent of this paper is not to provide a comprehensive review of PHS which has been well covered in several publications, *e.g.* refs. 12-16 but rather to provide evidence of involvement of oxidative stress in the etiology of this metabolic disease. This review will initially provide an overview of free radical and antioxidant biochemistry. Lipid peroxidation is initiated by free radicals and reactive oxygen species (ROS) while cellular defense against oxidative injury is provided by nonenzymatic (*e.g.* Vit C, E and glutathione [GSH]) and enzymatic (*e.g.* superoxide dismutase, catalase, GSH peroxidase) antioxidants. The reader should be aware of several excellent reviews which discuss this area in more detail^{4-9,17-19}. The paper will then describe physiological factors that may either predispose or promote the generation of free radicals and ROS in poultry. Evidence will be provided which indicates that lipid peroxidation, caused by free radicals generated during normal metabolism, by hypoxic-mediated biochemical events, or during toxic or inflammatory oxidative stress, may play a significant role in the etiology of PHS.

2 FREE RADICALS AND REACTIVE OXYGEN SPECIES

A. Definitions

Free radicals are chemicals that have one or more

unpaired electrons in their outer orbit. Many terms are used to describe free radicals containing oxygen, including oxyradicals and oxygen free radicals, and reactive oxygen species (ROS) which will be used throughout this review. Hydrogen peroxide, hypochlorous acid, hydroperoxide, epoxides and other compounds contain chemically reactive oxygen-containing functional groups and do not always interact with tissues through radical reactions⁵.

In the metabolism of oxygen (O₂), ROS or radicals are often produced. Molecular oxygen is actually a biradical because it has two unpaired electrons at different pi orbitals. When O₂ accepts a pair of electrons from another molecule during oxidation, the electron pairs must have parallel spins which force electron transfer to restricted antiparallel spins. Spin restriction sets up situations for one-electron transfers to occur which also enhances free radical formation²⁰. An outline of properties of ROS and the four basic types of electron transfers that can allow for oxygen radical formation is provided in Table 1.

If a radical is formed and not reduced by antioxidant protective mechanisms, tissue damage can ensue. Although the term oxidative stress has been used to denote tissue injury from chemical radicals, a problem with its broad and liberal usage in the literature has led to defining oxidative stress as a disturbance in the prooxidant-antioxidant balance leading to potential tissue damage⁵.

B. Oxygen radicals

1. *Superoxide radical*: In aerobic systems, O₂ is reduced to water in the electron transport chain (ETC) of mitochondria. While most O₂ is completely reduced to water, there is leakage of single electrons from complexes I, II and/or III of the electron transport chain leading to an incomplete reduction of O₂ to form the superoxide radical (O₂^{•-}). It has been estimated that as much as 2% of the oxygen consumed by mitochondria results in the formation of O₂^{•-} or hydrogen peroxide (H₂O₂)²¹. Although this 2% figure is often quoted, Kehrer (1993)⁵ has indicated that the experimental conditions utilized

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Role of free radicals

Table 1. Abbreviations, general properties and electron transfers required for production of major reactive oxygen species.

Species	Abbr.	Properties
Superoxide radical	$O_2^{\bullet-}$	Good reductant, poor oxidant
Hydroxyl radical	$\bullet OH$	Very reactive, low diffusion distance
Hydrogen Peroxide	H_2O_2	High diffusion distance, low reactivity
Peroxyl Radical	ROO^\bullet	Less reactive than $\bullet OH$, high diffusability
Alkoxy Radical	RO^\bullet	Intermediate lipid reactivity
Hypochlorous Acid	$HCIO$	
Nitrogen Dioxide Radical	NOO^\bullet	

One electron transfer equations:

superoxide radical	$O_2 + e^- \rightarrow O_2^{\bullet-}$
hydroperoxyl radical	$O_2 + H_2O \rightarrow HO_2^{\bullet-}$
hydrogen peroxide	$H_2O + e^- + H \rightarrow H_2O_2$
hydroxyl radical	$H_2O_2 + e^- \rightarrow OH^- + \bullet OH$

were highly artificial and actual amounts of free radicals produced *in vivo* would probably be much lower. Nonetheless, this value represents an estimate of the potential for free radical generation which might be obtained under pathological conditions. For example, the incomplete reduction of O_2 is accentuated in hypoxic conditions as the flavin adenine nucleotides remain reduced and unable to accept electrons.

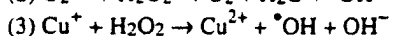
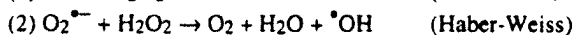
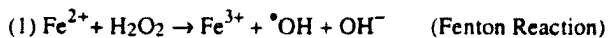
Besides mitochondria and other electron transport systems, $O_2^{\bullet-}$ is produced by several enzymes, notably NADPH oxidase, xanthine oxidase, amino acid oxidase, during epinephrine metabolism, and by the cytochrome P450 system. Singlet oxygen and hydroxyl radical are byproducts of arachidonate metabolism in prostaglandin synthesis catalyzed by cyclooxygenase. It has been estimated that a single cell in a living organism may be exposed to 10^{10} molecules of $O_2^{\bullet-}$ per day²² representing 1.75 kg of $O_2^{\bullet-}$ produced in a 70 kg human over a one year period⁴. This would roughly translate to a range of 2 to 5 gm of $O_2^{\bullet-}$ being produced in a broiler from 0 to 49 days of age.

2. Hydroxyl radical: The $\bullet OH$ is extremely reactive and considered the most potent oxidant in biological systems. Important biological sources of $\bullet OH$ arise from the

Table 2. Major antioxidant protective compounds or protective systems.

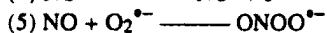
Type	Tissue Location	Action
I. Fat Soluble		
- Vitamin E (tocopherol)	Membranes, extracellular fluid	Converts $O_2^{\bullet-}$, $\bullet OH$, and lipid peroxyl radicals to less reactive compounds. Chain breaking antioxidant; prevents or protects against lipid peroxidation.
- Vitamin A (β -carotene)	Membranes	Scavenges $O_2^{\bullet-}$; Interacts directly with peroxyl radicals
II. Water Soluble		
- Vitamin C (ascorbic acid)	Widely distributed in intra- and extracellular fluid	Interacts directly with $O_2^{\bullet-}$, $\bullet OH$. Neutralizes ROS released from WBC's. Can regenerate Vit. E from E radical form.
- Glutathione	Mainly intracellular, low levels found in plasma	Interacts directly with $O_2^{\bullet-}$, $\bullet OH$ and lipid hydroperoxides. Serves as substrate for GSH recycling enzymes.
- Uric Acid	Widely distributed	Binds transition metals; interacts directly with $O_2^{\bullet-}$, $\bullet OH$, and peroxyl radicals; Spares or prevents oxidation of ascorbic acid.
- Cysteine	Widely distributed	SH is a strong reducing agent for many compounds; rate limiting amino acid for GSH synthesis.
- Glucose	"scavenges OH	
III. Enzymatic		
- Superoxide Dismutase		
Mn SOD	Mitochondria	converts $O_2^{\bullet-}$ to H_2O_2 by dismutation reaction
Cu SOD	Plasma	
Cu/Zn SOD	Cytosol and Nucleus	
- GSH recycling system		
GSH peroxidase	cytosol and mitochondria	reduces H_2O_2 and other hydroperoxides, low K_m . functions during 'normal' metabolism
GSH reductase	cytosol and mitochondria	reduces low molecular weight disulfides (GSSG—GSH) using NAD(P)H
- Catalase	peroxisomes	reduces H_2O_2 , high K_m . functions mainly in disease states

decomposition of hydrogen peroxide by the Fenton reaction (Eq. 1), by the interaction with superoxide and hydrogen peroxide through the Haber-Weiss reaction (Eq. 2) and by the interaction of transition metals with hydrogen peroxide (Eq. 3):



3. Hydrogen Peroxide: Hydrogen peroxide is an important biological oxidant due to its a) ability to interact with transition metals and form hydroxyl radical (Eqs 1-3), and b) lipid solubility with associated capacity to penetrate membranes (as shown in mitochondrial H_2O_2 leakage). Therefore, the biological importance of H_2O_2 is considered more as an initiator of free radical toxicity rather than as an oxidant.

4. Nitric Oxide radical and Peroxynitrite: Nitric Oxide (NO) has received much attention since the discovery of its vital function as the endothelium dependent relaxing factor in the vascular system²³⁻²⁵. NO has now been recognized as a product of macrophages that contributes to cell killing²⁶. Although technically not a reactive compound, NO can form an NO^\cdot radical (Eq. 4) or a peroxynitrite radical (ONOO^\cdot) by reacting with $\text{O}_2^{\cdot-}$ (Eq. 5) and contribute to the cell killing capability of macrophage cells.



Nitric oxide synthase (NOS) converts arginine to citrulline and NO. It was suggested by Dietert and Golemboski²⁷ that arginine requirements of chicks and other animals to support adequate growth may not be adequate for optimal cytotoxic activity of macrophages. In a study by Taylor *et al.* (1992)²⁸, chicks fed an arginine-supplemented diet exhibited smaller tumors produced by Raus sarcoma virus that was associated with improved immune function than did birds receiving a control (arginine-adequate) diet. The results of this study indicated that higher levels of dietary arginine may have attenuated tumor growth through enhanced phagocytic cytotoxicity²⁷.

Because it is lipid soluble and able to react with lipid radicals, NO may also possess antioxidant capabilities. It was observed that depletion of α -tocopherol *in vitro*, coinciding with the formation of lipid peroxyl radical, was prevented by the presence of an NO generating system provided by s-nitrosoglutathione (GSNO)²⁹.

C. Cellular sources of free radicals.

1. Phagocytes: A major function of macrophage and lymphocytic cells is to clear foreign substances from the body. When activated, phagocytic cells consume oxygen

in an oxidative burst that results in a dramatic increase in the ROS being produced³⁰. Superoxide is formed through the action of NADPH oxidase activity while myeloperoxidase utilizes hydrogen peroxide and halides such as chlorine in the formation of hypochlorous acid³¹. Free radicals are not only vital for cell killing but play an important role in generating chemotactic factors, such as cytokines and prostaglandins, that are required in an inflammatory response³² and possibly in signal transduction pathways. As indicated above, the extracellular formation of NO^\cdot and ONOO^\cdot contribute to cell killing by phagocytes. Excessive free radical generation during inflammation can cause damage to surrounding tissues.

Because of its contact with a variety of environmental pollutants and microbes, and high oxygen levels, the lung is particularly prone to free radical-induced oxidant injury. Free radicals and ROS can be generated in the lower respiratory tract by phagocytes involved in local inflammatory reactions³³⁻³⁵ and have been recognized as important factors in the etiology of various lung diseases^{1,35,36}. Protection against free radical induced injury from inflammation or toxins is provided by a host of antioxidants including vitamin E, vitamin C and glutathione³⁶⁻³⁹.

2. Electron transport systems; Mitochondria: Free radicals are produced in mitochondrial and microsomal electron transport systems. As much as 2% of total oxygen consumption by mitochondria may be incompletely reduced to $\text{O}_2^{\cdot-}$ and H_2O_2 ²¹. During toxic insult, inflammation, hypoxia, high rates of metabolism, or with antioxidant deficiency, ROS generated within mitochondria can exceed its antioxidant protective capacity. ROS which escape degradation can then initiate lipid peroxidation and severely compromise cell function. Normally, these reactive metabolites are converted (reduced) to harmless compounds by GSH, GSH peroxidase and superoxide dismutase (SOD) within mitochondria. The endoplasmic reticulum contains several types of monooxygenases which specifically oxidize compounds in the detoxification of xenobiotics. As in mitochondria, ROS leakage during this process can cause damage to surrounding structures.

3. Oxidases: There are a number of enzymes that oxidize endogenous and exogenous substrates. One of the best known is xanthine oxidase which can reduce molecular oxygen to superoxide and hydrogen peroxide. Other oxidases include fatty acyl CoA oxidase, D-amino oxidase, dopamine β -hydroxylase but their contribution to free radical release and consequent tissue injury is not known. Cyclooxygenase also produces superoxide and lipid hydroperoxides.

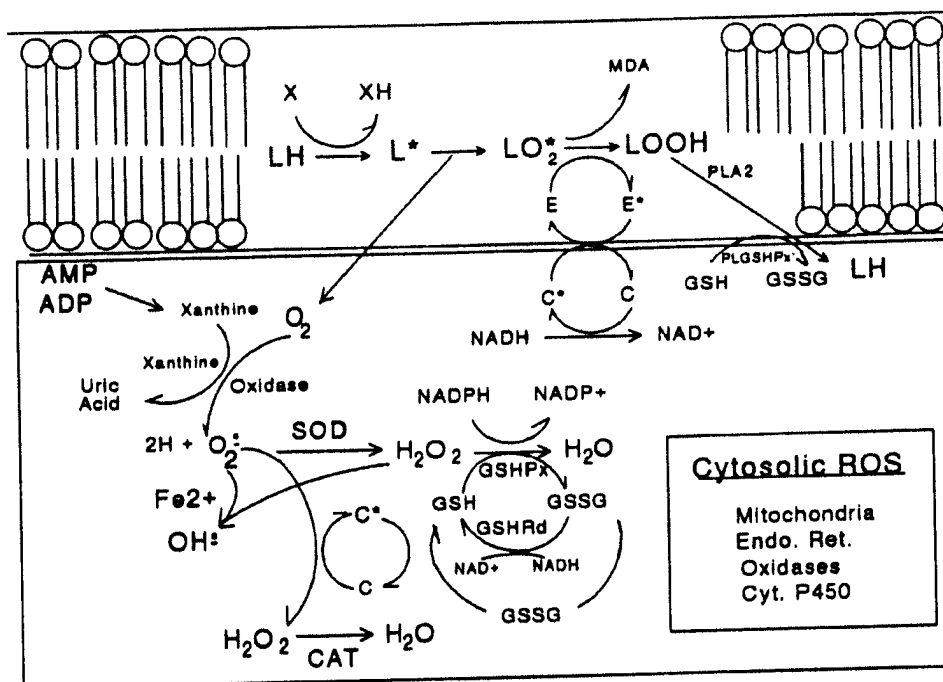
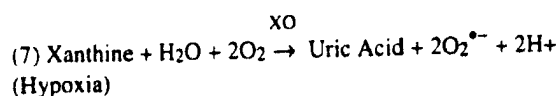
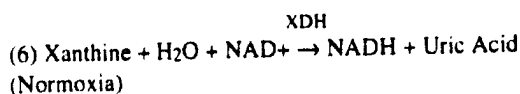


Fig. 1. Summary of cellular antioxidant protective mechanisms; see text for details.

3 ANTIOXIDANT PROTECTIVE MECHANISMS

Xanthine oxidase may play a particularly important role in PHS by virtue of its ability to generate $O_2^{\bullet-}$. During hypoxic conditions, there is a stimulation of anaerobic glycolysis with enhanced adenylate degradation^{40,41}, leading to increased concentrations of purine metabolites, such as hypoxanthine, which can serve as substrate for both xanthine dehydrogenase and xanthine oxidase. Under normoxia, xanthine dehydrogenase (XDH) catalyses the conversion of xanthine to uric acid with the transfer of hydrogen to NAD^+ (Eq. 6), but with hypoxia, xanthine dehydrogenase is converted to xanthine oxidase⁴² (XO) which catalyses the catabolism of xanthine to uric acid with the concomitant formation of superoxide radical (Eq. 7)⁴³.



The antioxidant systems responsible for protecting cells from the actions of free radicals and ROS are diverse, intertwined and many layered. A list of some of the major antioxidant compounds and enzymatic systems is provided in Table 2 with a summary of activities provided in Figure 1. These protective compounds are strategically located in organelles, subcellular compartments, or extracellular space to afford maximum cellular protection. For example, MnSOD and GSH peroxidase (GSHPx) are situated within mitochondria where most of the intracellular free radical production occurs²¹ as well as the cytosol. The cooperative interaction between antioxidants in the plasma is vital for maximum protection from the deleterious effects of extracellular free radicals. Antioxidants are classified as being enzymatic or nonenzymatic with protection associated with primary defenses, including antioxidant compounds and scavenging enzymes, or secondary defenses which include lipases, proteases, peptidases that repair or excise damaged portions of molecules following oxidative stress⁴⁴. The focus of this paper will be on the primary defense mechanisms; i.e. antioxidant enzymes and compounds and not on repair mechanisms associated with secondary defense against oxidative injury. It should also be recognized that there are many other antioxidants

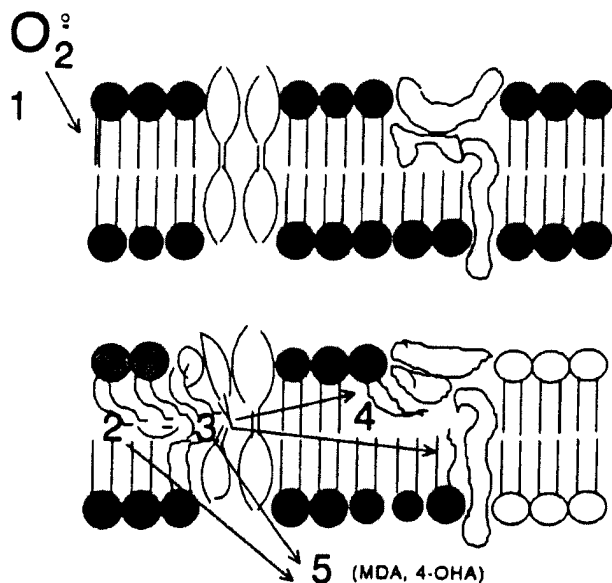


Fig. 2. Events involved in lipid peroxidation of membranes. (See text for details).

- 1) Free radical abstraction of an electron from a fatty acid causes formation of a peroxyl radical.
- 2) The peroxyl radical abstracts electrons from adjacent molecules
- 3) Deformation of membrane structure or oxidation of membrane associated proteins
- 4) Radicals formed during oxidation can be carried and damage distal portions of the membrane while
- 5) Malondialdehyde (MDA) and 4-hydroxyalkenal (4-OHA) release from the membrane can oxidize intracellular structures.

within the cell than those described below. Many of the components of the mitochondrial electron transport chain, for example ubiquinol and cytochrome C oxidoreductase, have strong reducing or antioxidant capabilities. The ones that are discussed below are potentially the most well known and characterized antioxidants of the cell.

A. Nonenzymatic - lipophilic

1. **Vitamin E:** Vitamin E has been recognized for some time as a major lipid soluble antioxidant⁴⁵. Vitamin E actually refers to a group of structural isomers of tocopherol of which α -tocopherol is the best known, possessing roughly 90% of the antioxidant activity of vitamin E^{46,47}. Vitamin E has a high lipid solubility and is located in plasma and organelle membranes; e.g. mitochondria and endoplasmic reticulum. Located within membranes with potent antioxidant capability, vitamin E is considered the major chain breaking antioxidant that

scavenges peroxyl and alkoxyl radicals and prevents further peroxidative damage of membranes. Vitamin E is essential in maintaining membrane integrity of the cardiovascular, nervous and immune systems. Tocopherol deficiency is associated with a number of chronic health problems and causes severe membrane damage from oxidative stress⁴⁷. Conversely, supplementation of vitamin E above recommended levels in animals improved resistance to microbial infections⁴⁸ apparently through an improved immune function such as demonstrated in mice⁴⁹ and chicks⁵⁰.

2. **Vitamin A:** β -carotene and other carotenoids protect lipids from lipid peroxidation by scavenging free radicals and other reactive oxygen species, especially singlet oxygen^{51,52}. Singlet oxygen is a reactive compound produced during photosensitization in the skin and eye. β -carotene exerts its antioxidant activity only at lower partial O_2 pressures similar to that found in the tissue⁵³. At higher oxygen levels, β -carotene loses its antioxidant capability and may actually become a prooxidant, similar to ascorbic acid as discussed below. The antioxidant capabilities of β -carotene are also weak in comparison to α -tocopherol⁴.

B. Nonenzymatic - hydrophilic.

1. **Vitamin C:** Vitamin C or ascorbic acid, is a hydrophilic antioxidant which reacts directly with $O_2^{\bullet-}$ and $\bullet OH$ as well as various lipid hydroperoxides⁵⁴. Ascorbate has been shown to function in restoring the antioxidant capabilities of oxidized vitamin E or by converting the tocopherol radical back to its reduced state⁵⁵. Prevention of *in vitro* lipid peroxidation in plasma by ascorbate was clearly demonstrated by Frei *et al.* (1988)⁵⁶ in which ascorbate activity disappeared more rapidly than other plasma antioxidants, notably sulfhydryl groups, bilirubin, urate, and α -tocopherol and this disappearance coincided with the onset of hydroperoxide formation. Excess ascorbate can have prooxidant consequences by reducing Fe^{3+} to Fe^{2+} which can then generate free radicals via the Fenton reaction (Eq. 1) or by interaction with Cu^{2+} (Eq. 3). Interestingly, microsomal lipid peroxidation was enhanced by providing ascorbate to tocopherol deficient animals but was prevented or attenuated when microsomes were isolated from animals fed diets with sufficient tocopherol¹⁸.

In poultry, ascorbic acid may be effective in lowering heat-stress mortality in broiler females⁵⁷. The performance of chickens, however is not always improved if dietary intake of ascorbic acid is increased⁵⁷⁻⁵⁹. Increased levels of ascorbic acid were effective in lowering ascites mortality induced by feeding broilers toxic levels of sodium chloride (2.5%)⁶⁰. It was suggested that part of the beneficial effect of vitamin C might be due

to its ability to enhance carnitine required for transport of long chain fatty acids into the mitochondria, but the authors⁶⁰ did not elaborate as to how enhancement of fatty acid movement into mitochondria, presumably to serve as an energy source, and PHS mortality were related. Birds fed higher levels of ascorbic acid also had higher levels of plasma protein which can function as a weak antioxidant. Hematocrit was also elevated with increased ascorbic acid levels which would in turn increase oxygen carrying capacity of the blood.

2. *Glutathione (GSH)*: Glutathione (GSH) is a ubiquitous tripeptide consisting of glutamate, cysteine and glycine. GSH has numerous vital homeostatic roles including protection of cells from oxidative injury⁶¹⁻⁶⁴. Reduced GSH contains a reactive thiol group and a γ -glutamyl bond which makes it resistant to degradation by all peptidases except γ -glutamyl transpeptidase. GSH donates reducing equivalents to numerous electrophiles and ROS including $O_2^{\bullet-}$, H_2O_2 and $\bullet OH$. The regeneration of ascorbic acid from dehydroascorbate can be facilitated nonenzymatically in tissues that have an excess of GSH^{65,66}. A decrease in tissue GSH could potentially compromise tissue antioxidant capacity directly or indirectly by affecting reduced ascorbate levels. Selective mitochondrial GSH depletion causes severe toxicity⁶⁷. Loss of mitochondrial GSH during oxidative stress leads to lipid peroxidation of mitochondrial membranes with subsequent disruption of Ca^{2+} regulation, is regarded as a key event in cell death^{68,69}. GSH also serves an antioxidant function by its association with GSH peroxidase and is involved in phase II detoxification by conjugation reactions catalysed by GSH-S-transferases. Oxidized GSH (GSSG) is formed following GSH peroxidase catalysed hydrogen transfer from GSH to lipid or hydrogen peroxides. Normally, GSSG levels are maintained at 1-2% that of GSH by GSH reductase activity but GSSG elevation is cytotoxic and indicative of oxidative stress in cells⁷⁰ and mitochondria⁷¹. Additional description of GSH metabolism and its relevance to PHS syndrome in broilers is provided below.

In mammals, the highest levels of GSH are found in the liver which provides GSH to other tissues by interorgan circulation^{61,72}. GSH is exported into the sinusoids into the general circulation and is then transported into the cell by γ -glutamyl transpeptidase. GSH is then resynthesized in the cell by GSH synthetic enzymes γ -glutamylcysteine synthetase and glutathione synthase with energy input from ATP⁶¹.

3. *Uric acid*: One of the earliest reports of antioxidant properties of uric acid was provided by Ames *et al.*⁷³. Uric acid may function by complexing iron or copper and thereby decreasing hydroxyl radical formation as well as sparing ascorbic acid. However, the production of uric

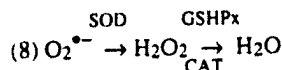
acid by xanthine oxidase may also be associated with increased ROS generation under hypoxic conditions⁴³.

4. *Others*: As mentioned previously, there are numerous compounds in the cell that possess antioxidant capabilities. Virtually all components of the electron transport chain can act as antioxidants; e.g. cytochrome C oxidoreductase, co-enzyme Q, ubiquinol¹⁹.

Although technically not an antioxidant, selenium nonetheless has been associated with antioxidants since it functions as a cofactor for GSH peroxidase⁷⁴. Selenium may also facilitate antioxidant capacity in the body indirectly by increasing the availability of cysteine for GSH synthesis via the transsulfation pathway which is responsible for the conversion of methionine to cysteine⁷⁵. Halpin and Baker⁷⁶ observed that transsulfation could be impaired in some strains of chicks by selenium deficiency.

C. Enzymatic

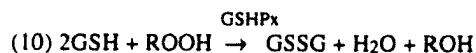
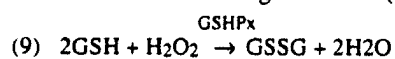
1. *Superoxide dismutase (SOD)*: As shown in Table 2, there are 3 forms of SOD; MnSOD, found primarily within mitochondria, CuSOD, found primarily in plasma and extracellular fluid, and Cu/Zn SOD which is found primarily in the cytosol as well as the cell nucleus. The importance of CuSOD as an antioxidant has often been dismissed or overlooked because of its extracellular location. However, high Cu/Zn SOD activity has been found in lungs. The activity of CuSOD was maintained for over 100 hours through anchoring of the molecule by heparin sulfate to epithelial and endothelial cell membranes⁷⁷. All forms of SOD catalyze the dismutation of $O_2^{\bullet-}$ to H_2O_2 . SOD functions in conjunction with catalase and GSH peroxidase to catabolize oxidizing ROS generated in the cell (Eq. 8). The highest activity of SOD is found in liver, adrenal gland, kidney and spleen. SOD activity is also high in erythrocytes.



2. *Catalase (CAT)*: Catalase and GSH peroxidase both catalyze the reduction of H_2O_2 to water but because of different cellular locations and affinities for H_2O_2 , their importance varies depending on metabolic conditions. Due to a higher K_m , and a primary locus within peroxisomes, catalase is believed to serve a more important role in protection during disease states; e.g. inflammation with increased H_2O_2 production or when the supply of NADPH is limiting during oxidative stress⁷⁸. Conversely, GSH peroxidase has a much lower K_m and is located in both the cytosol and mitochondria. Consequently, GSH peroxidase is believed to function in the reduction of ROS generated during normal metabolism. These enzymes also differ in substrate

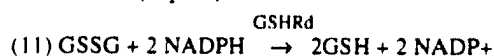
specificity as catalase reacts only with H_2O_2 whereas GSH peroxidase reacts with lipid hydroperoxides as well as H_2O_2 . Because H_2O_2 can move throughout the cell and be converted to the $\cdot\text{OH}$ radical in the presence of transition metals (Eqs 1-3), catalase and GSH peroxidase serve a vital function by lowering H_2O_2 availability for $\cdot\text{OH}$ radical production.

3. *GSH recycling enzymes; GSH peroxidase and GSH reductase:* GSH peroxidase reduces H_2O_2 (Eq. 9) and organic hydroperoxides (Eq. 10) to water or lipid alcohols by donating a hydrogen from the sulfur group of the cysteinyl moiety. In so doing, two GSH molecules combine to form oxidized glutathione (GSSG).



GSH peroxidase has been designated as either selenium dependent or selenium independent GSH peroxidase that differ in affinity for H_2O_2 and organic hydroperoxides. Recently, a third form of GSH peroxidase has been reported that is also a selenoenzyme that reduces phospholipid hydroperoxides⁷⁹. Unlike the other forms of GSH peroxidase, this PLGSH peroxidase interacts directly with peroxidized lipids even when associated with biomembranes whereas GSH peroxidase reduces lipid hydroperoxides only following their release from membranes by phospholipases⁸⁰.

Reduction of GSSG is vital to cell function as elevations in GSSG can oxidize proteins through protein sulfhydryl formation. Increased GSSG represents an effective loss of GSH and therefore a lowering of antioxidant capability to the cell. GSH reductase (GSHRd) utilizes reducing equivalents from NADPH produced by the hexose mono-phosphate shunt to reduce GSSG to GSH (Eq. 11).



Normally, GSH reductase activity maintains cellular GSSG levels low (1-5% of total GSH). However, with high GSH peroxidase activity that occurs during oxidative stress or hypoxia, when reducing equivalents required for GSH reductase activity become limiting, GSSG is actively transported out of many cell types in order to maintain cell redox state and prevent protein disulfide formation^{81,82}. An inverse relationship between GSH-peroxidase activity and oxidant injury indicates GSH peroxidase is a key enzyme in cell protection; any decrease in activity renders cells more susceptible to oxidative stress⁸³. GSH peroxidase and MnSOD are particularly vital for maintaining mitochondrial function by preventing lipid peroxidation that occurs from the generation of superoxide and H_2O_2 .

D. Summary of antioxidant protective mechanisms.

A summary of cellular antioxidants protecting against free radical-mediated injury is provided in Figure 1. Depicted at the top of the figure is a lipid bilayer where abstraction of an electron from a lipid (LH) by a free radical (X) generates a lipid peroxyl radical ($\text{L}\cdot$). With the addition of oxygen, a lipid peroxide ($\text{LOO}\cdot$) is formed with the concomitant release of malondialdehyde (MDA). Vitamin E (E) reduces the lipid hydroperoxide to a lipid alcohol (LOOH) that is released from the membrane by phospholipase. Once released, the lipid hydroperoxide can be converted to an alcohol by GSH peroxidase. In reducing the lipid hydroperoxide, vitamin E is converted to a tocopherol radical ($\text{E}\cdot$). Movement of ascorbic acid (C) in close proximity to the lipid-cytosol interface allows the regeneration of reduced vitamin E to occur. The dehydroascorbate (DA) can be reduced back to ascorbate through reducing equivalents provided by NADH.

Cytosolic components of the antioxidant protective mechanisms are represented in the bottom enclosed portion of Figure 1. The conversion of xanthines and hypoxanthines by xanthine oxidase under hypoxic conditions produces uric acid and $\text{O}_2^{\cdot-}$. Superoxide is converted to H_2O_2 by SOD and then reduced to water by either GSH peroxidase or catalase. In the presence of transition metals, $\text{O}_2^{\cdot-}$ and H_2O_2 are converted to the highly reactive $\cdot\text{OH}$ which highlights the important antioxidant role that the concerted activities of SOD, GSH peroxidase and catalase have in metabolizing free radicals and ROS. The GSSG formed during this process is reduced to GSH by GSH reductase using reducing equivalents from NADPH produced by the pentose-phosphate shunt. There is also some evidence that GSSG may be reduced to GSH by vitamin C *in vitro*. Vitamin C is also important in free radical scavenging and can reduce $\text{O}_2^{\cdot-}$ to H_2O_2 .

E. Consequences of lipid peroxidation

Lipid peroxidation can have numerous detrimental effects on cell function¹⁷. The process of lipid peroxidation, depicted schematically in Fig. 2, is a chain reaction event in which formation of one peroxyl radical can trigger subsequent oxidation and radical formation. Peroxylipid radicals formed by interactions of free radicals with polyunsaturated fatty acids (PUFA) can abstract electrons from neighboring fatty acids as well as membrane associated proteins. Crosslinking of proteins following the release of MDA can inactivate membrane bound enzymes and physical changes within the membrane can alter tertiary structure of membrane proteins. For example, there is evidence that Na^+/K^+ ATPase activity is disrupted by lipid peroxidation^{84,85}; such disruption could contribute to an inability of membranes to maintain

the electrical gradient required for cell viability. Increases in cytosolic Ca^{2+} following oxidative induced damage to Ca^{2+} -ATPase causes the loss of cytosolic Ca^{2+} regulation and has been suggested as a key step leading to cell death⁸⁶. Free radicals can also oxidize proteins and DNA within the cell, while extracellular release of ROS can damage neighboring cells. Another group of toxic compounds formed during lipid peroxidation are the 4-hydroxyalkenals which react with sulfhydryl groups of proteins, and along with MDA, can convey the toxic effects of lipid peroxidation throughout the cell. In mitochondria, lipid peroxidation diminishes electron transport capability which can generate more free radicals. Peroxidation of erythrocytes and lysosomal membranes causes cell or organelle lysis.

It was suggested by Squires and Summers⁸⁷ that lipid peroxidation might play a role in the etiology of PHS. Sudden death syndrome (SDS), in which birds die of sudden cardiovascular failure, has many characteristics similar to PHS and may simply be an earlier manifestation of PHS; *i.e.* birds that are most susceptible to PHS die early from cardiovascular failure. Squires and Summers⁸⁷ hypothesized that decreased SDS mortality observed when birds were fed animal fat⁸⁸ might have been due to lowered intake of polyunsaturated fatty acids which are more susceptible to lipid peroxidation. It should also be pointed out that under certain conditions saturated fatty acids may facilitate, and unsaturated fatty acids diminish the absorption of lipid soluble antioxidant vitamins⁸⁹. Thus, the higher incidence of sudden death in birds fed higher levels of unsaturated fats could be from increased susceptibility to oxidative stress compounded by decreased absorption of vitamin E and A needed to protect membranes from oxidative insult.

Besides disrupting cell function, H_2O_2 and other lipid hydroperoxides cause vaso- and bronchoconstriction via synthesis and release of arachidonic acid metabolites⁹⁰. Thromboxane (TxA_2) is a powerful vasoconstrictor known to play a role in peroxide-induced lung injury^{90,91}. Prostacyclin (PGI_2) is a powerful vasodilator synthesized in endothelial cells and opposes the actions of TxA_2 . Prostacyclin synthase is sensitive to lipid peroxides and is inactivated when antioxidant levels are inadequate⁹². Buckley *et al.* (1991)⁹³ demonstrated that PGI_2 was depressed by high levels of H_2O_2 in endothelial cell culture. Thus, a shift from PGI_2 to TxA_2 synthesis in response to lipid peroxidation might also contribute to the development of PHS in broilers.

4 FREE RADICALS AND ANTIOXIDANTS IN PHS ETIOLOGY

A. Overview

Mortality rates of 2 to 5% from PHS are common and estimated to cost the US poultry industry in excess of \$100 million annually⁹⁴. Several reviews and reports have described factors that contribute to the onset of PHS such as high altitude hypoxia, poor ventilation, cold, stress and fast growth rate^{12,14-16}.

A major physiological factor contributing to PHS is a marginal cardio-pulmonary capacity in broilers that leads to the development of a progressive systemic hypoxia. Evidence for a basic pulmonary inadequacy in avian lungs has been reviewed⁹⁵ and is derived from research indicating;

- 1) smaller lungs per unit of body weight with a thicker blood gas barrier and lower oxygen diffusing capacity in single comb white leghorn fowl compared to red jungle fowl⁹⁶,
- 2) lower lung and gas exchange volumes as well as lower air and blood capillary surface area in selected as opposed to unselected turkey lines^{97,98}, and
- 3) less compliant pulmonary vasculature in duck and goose than in mammals⁹⁹.

The latter aspect pertaining to a relative noncompliant nature of the avian pulmonary vasculature highlights the fact that even small increments in cardiac output can increase pulmonary arterial pressure. The cardiopulmonary inadequacy would lead to a ventilatory-perfusion mismatch and the development of systemic hypoxia.

The sequelae of physiological events leading to PHS and congestive heart failure has been outlined⁹⁵ and summarized as follows. With increased metabolic rate and development of systemic hypoxia, vasodilation in peripheral tissues increases venous return to the heart. With increased venous return, there is a requisite increase in cardiac output pumped into pulmonary vasculature already maximally distended, the consequence of which is an increase in pulmonary arterial pressure (pulmonary hypertension). For the increased blood volume to move through the pulmonary vasculature, transit time decreases, allowing less time for gas exchange, resulting in progressive hypoxia. As pulmonary arterial pressure increases during PHS, right ventricular hypertrophy occurs. The ratio of the right ventricular to total ventricular weight has been used as a reliable indicator of the presence and magnitude of pulmonary hypertension. As the heart is now unable to pump all the blood returning to it, central venous pressure rises. This increase in venous pressure is conveyed to the hepatic vasculature where fenestrated endothelium allows plasma to be forced

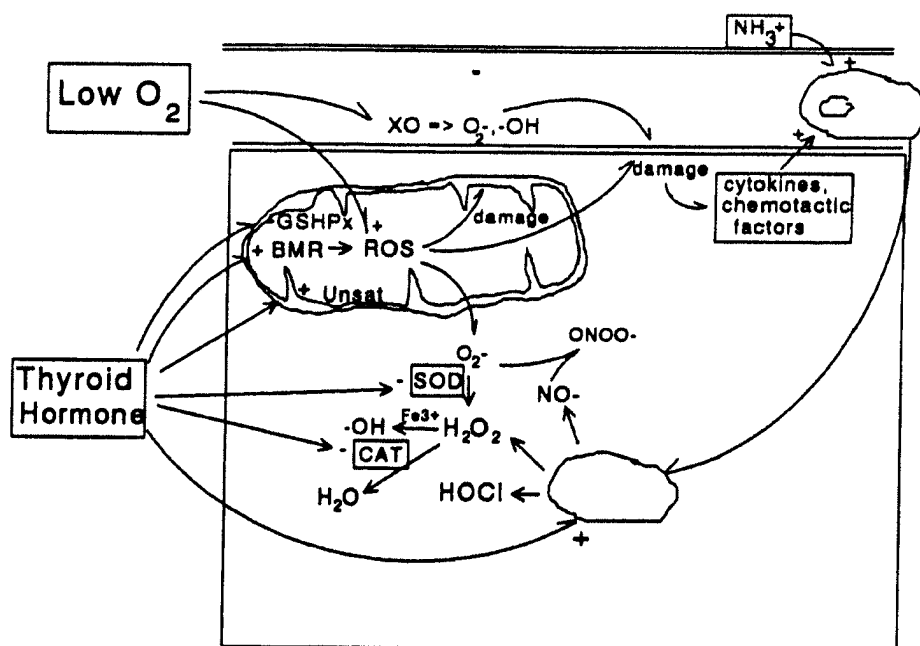


Fig. 3. The effects of low oxygen (hypoxia) and thyroxine on cellular events that could lead to increased free radical production in cells. In addition, ammonia and dust (not shown) potentially contribute to free radical production by activation of lymphocytes. (See text for more details).

from the hepatic sinusoids and exuded from the surface of the liver. At this stage, the presence of ascites fluid can be detected in the body cavity. The developing vascular congestion can be detected by cyanosis of the comb and wattles and venous congestion in superficial vessels. Ventricular hypertrophy leads to incompetency of the right atrio-ventricular monocuspid valve and ultimately leads to congestive heart failure.

While much is known about PHS, the exact biochemical mechanisms responsible for producing the disease have not been identified. As will be discussed below, there is evidence that free radical mediated mechanisms may be involved in the etiology of PHS. Increased incidence of PHS frequently associated with cold weather, rapid growth, and high altitude, may have a commonality attributed to increased free radical generation and/or an inability of existing endogenous antioxidants to cope with the amount of ROS being produced. Possible contributions to free radical production and/or altered antioxidant capability by systemic hypoxia, inflammation, and thyroid hormone will be described.

B. Potential factors contributing to peroxidation during PHS

1. Thyroid hormone: Thyroid hormone could play a major role in the etiology of PHS. In a thorough review of the literature, May¹⁰⁰ indicated that avian and mammalian thyroid hormone metabolism are qualitatively similar. Particularly relevant to PHS is evidence that high levels of feed intake and low ambient temperatures increase circulating levels of thyroxine. Scheele *et al.*¹⁰¹ concluded that diets producing hypothyroid-like conditions could lower PHS in broilers and hypothesized higher incidences of PHS mortality would occur with hyperthyroid conditions. This hypothesis was supported by Buys *et al.*¹⁰² who reported that 1 ppm dietary T₃ accentuated PHS symptoms; *i.e.* increased ascites fluid accumulation, right ventricular hypertrophy, and mortality.

A major function of thyroid hormone is to increase energy metabolism especially in support of rapid rates of growth¹⁰³, a major selection criteria in broilers and often associated with high PHS mortality. Thyroid hormone treatment increases oxidative stress presumably by increasing basal metabolic rate^{104,105}. Increased cellular respiration mediated by thyroxine, caused a decline in mitochondrial GSH that was associated with an increase in mitochondrial lipid peroxidation¹⁰⁶. In the liver,

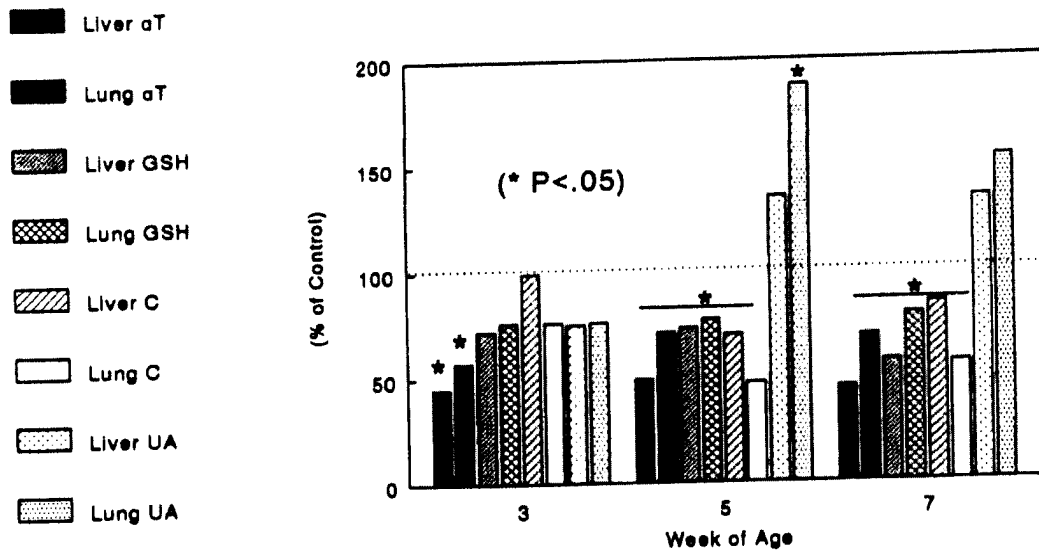


Fig. 4. Tissue concentrations of α -tocopherol (aT), reduced glutathione (GSH), ascorbic acid (C) and uric acid (UA) in the liver and lung at 3, 5 and 7 weeks of age in broilers with fulminant symptoms of pulmonary hypertension syndrome induced by low ventilation conditions. Values are expressed as a percent of tissue values in control broilers and calculated from values presented by Enkvetchakul et al. (1993)¹⁰. An asterisk (*) indicates values were different from control ($P < .05$). Only α -tocopherol values were significantly lower at 3 weeks of age whereas at 5 and 7 weeks of age, α -tocopherol, ascorbic acid and GSH were low in liver and lung in broilers with PHS. Uric acid levels in lung tissue and plasma (not shown) were elevated in broilers with PHS.

thyroid hormone lowered hepatic GSH¹⁰⁷ and increased superoxide generation and lipid peroxidation by enhancing microsomal and mitochondrial electron transport activity^{104,105,108}. Since hyperthyroidism decreased hepatocyte GSH concentrations and activities of SOD and catalase¹⁰⁵, increased basal metabolic rate seems to augment tissue lipid peroxidation potential as hypothesized by Cutler (1985)¹⁰⁹. Three days of thyroid hormone treatment, also increased unsaturation of inner mitochondrial membrane lipids¹¹⁰. Because polyunsaturated fatty acids are more susceptible to oxidation¹⁷, the combination of lower mitochondrial GSH needed to protect membranes, and a higher degree of mitochondrial lipid unsaturation, would seemingly predispose hyperthyroid animals to peroxidative insult. Morini et al. (1991)¹¹¹ demonstrated that the activities of GSH recycling enzymes (GSH reductase and GSH peroxidase) were increased in mitochondria of hyperthyroid mice. The increased activity of these enzymes would be necessary to protect the mitochondria from lipid peroxidation. Conversely, if the activity of these enzymes had not increased, then oxidative damage

to membranes might have occurred. Possibly, PHS susceptible birds tend to be hyperthyroid, but lack sufficient antioxidant protective mechanisms resulting in an increased potential for free radical generation. Such a scenario might have accounted for the increase in plasma lipid peroxides observed in birds with PHS¹¹.
2. Inflammation: Maxwell et al.¹¹² provided histological evidence of inflammatory cell infiltration throughout tissues of birds from field cases of PHS. Similar observations were made in birds with PHS induced by cool temperature or low ventilation conditions¹¹³. It has been known for many years that dust and ammonia can activate white blood cells to infiltrate tissue. As PHS often increases during periods of cool or cold weather when ventilation is reduced to save on gas expenditure, dust and ammonia buildup could initiate an inflammatory response with the concomitant release of high amounts of H₂O₂ and hypochlorous acid following a respiratory burst which aid phagocytes in clearing foreign material^{30,114}. The respiratory burst in phagocytes may also be linked to thyroid hormone as respiratory burst activity is elevated during hyperthyroidism¹¹⁵. While inflammatory

mediators are usually confined, damage to surrounding cells often occurs.

3. Hypoxia: Phagocytic cell infiltration also occurs when tissues have been exposed to low oxygen levels, followed by reintroduction of adequate oxygen; i.e. ischemia-reperfusion injury. Free radical and ROS release following white blood cell infiltration of tissue has been hypothesized as a key event in ischemia-reperfusion injury, especially in liver¹¹⁶⁻¹¹⁹, heart¹²⁰⁻¹²² and intestinal tissues^{123,124}. In the heart, a free radical-mediated decrease in creatine kinase activity following periods of ischemia may contribute to cardiac dysfunction¹²⁵. Organs in birds with PHS may experience situations similar to ischemia-reperfusion injury as systemic hypoxia develops as a consequence of a basic cardiopulmonary inadequacy^{96,126,127}. Fluctuations in tissue oxygenation, which are particularly damaging to mitochondrial function (see below), could be hypothesized to occur in birds with PHS resulting not only in fluctuations of tissue oxygenation but also in transient changes of oxidative metabolism. Whether inflammation during PHS¹¹² is a consequence of an ischemia-reperfusion-like injury or due to inflammatory cell activation following contact with dust and ammonia as recently hypothesized¹⁰ is not known.

Hypoxic tissue injury from ROS generated during relative, but not absolute lack of oxygen, can cause severe organ damage and is particularly damaging to mitochondria¹²⁸. Dawson *et al.* (1993)¹²⁸ indicated that reductive stress from relative, but not absolute hypoxia was very detrimental to mitochondrial function and suggested that "...under conditions of low-(blood) flow or intermittent ischemia, cycles of anoxia and reoxygenation may be occurring almost continuously, leading to increased reactive oxygen species and more tissue injury." Thus, inadequate pulmonary oxygenation in PHS may lead to increased free radical generation and lipid peroxidation, especially in mitochondria. Organs with high metabolic rates (e.g. liver, heart, intestines, kidneys) would be particularly susceptible to systemic hypoxia. If the rate of ROS formation in mitochondria exceeds the antioxidant protective capacities afforded by mitochondrial MnSOD and GSH peroxidase activities, then mitochondrial dysfunction may occur with free radicals being produced capable of damaging other parts of the cell. The presence of GSH and GSH peroxidase in mitochondria normally prevents peroxidation, but during oxidative stress (e.g. toxic insult, inflammation, or high metabolic rate) or with antioxidant deficiency, ROS can escape degradation and initiate lipid peroxidation. An inverse relationship between mitochondrial GSH and lipid peroxidation has been reported¹⁰⁶. Since ROS stimulate neutrophil infiltration^{124,129}, the general inflammation in

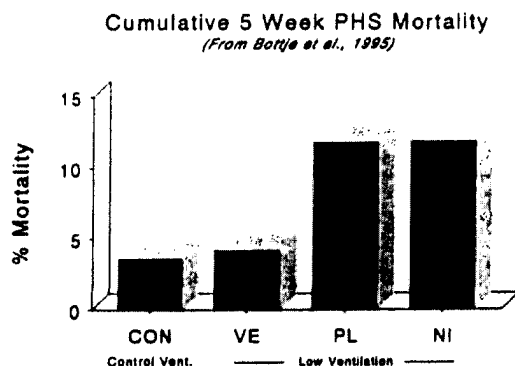


Fig. 5. Mortality due to PHS in broilers maintained under control conditions (CON) and in broilers maintained under low ventilation conditions that were provided a vitamin E implant (VE), a placebo implant (PL) or were not implanted (NI). The birds were implanted at 1 d. The vitamin E implant released a total of 15 mg of α -tocopherol at a constant rate over a 21 day period. (From Bottje *et al.* 1995¹¹).

birds with PHS^{112,113} may follow an hypoxic-induced increase in ROS generation within mitochondria, and/or other sites within the cell.

Free radical damage during ischemia-reperfusion injury has been partially attributed to the release of xanthine oxidase from liver and intestines into the circulation¹³⁰. Binding of xanthine oxidase to endothelial cells elevates the concentrations of superoxide, OH^{\bullet} , and uric acid on the surface of endothelial cells and may initiate peroxidative damage distal to the initial injury site^{17,130}. Hypoxanthine, the substrate of xanthine oxidase, is formed from purines (e.g. ADP, AMP) which accumulate during hypoxia. Indirect evidence that increased xanthine oxidase activity might be involved in the development of PHS stems from studies demonstrating urate accumulation in tissues^{10,112} and plasma¹⁰ of birds with PHS.

Figure 3 depicts the combined contributions that hypoxia and thyroid hormone could potentially have on free radical production in cells. Lymphocytic infiltration of tissues is stimulated cytokines and chemotactic factors released from damaged cells as well as by dust and ammonia in the atmosphere. Once inside the cell, the respiratory burst releases NO^{\bullet} , HOCl , H_2O_2 , and OH^{\bullet} . The respiratory burst is amplified in the presence of thyroid hormone. Thyroid hormone also diminishes antioxidant capacity in the cell as well as increasing free radical production via increases in basal metabolic rate (BMR). Increased amounts of superoxide and OH^{\bullet} via the action of xanthine oxidase cause additional damage to tissue endothelial cells.

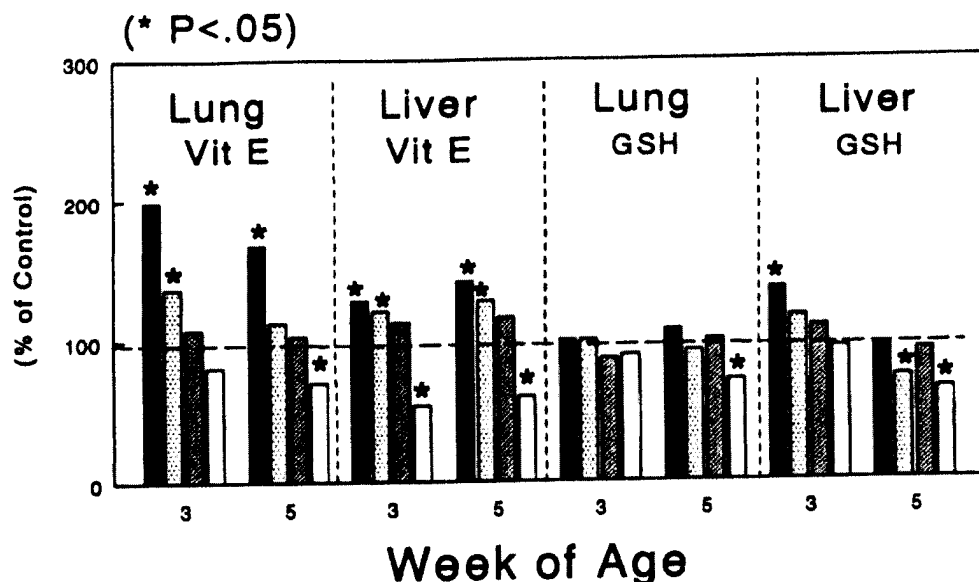


Fig. 6. Lung and liver vitamin E (Vit. E) and reduced glutathione (GSH) concentrations in broilers receiving a 3 week vitamin E implant (VE, solid bar), a placebo implant (PL, dotted bar), or that were non-implanted (NI, cross hatched bar), and in broilers with fulminant pulmonary hypertension syndrome (PHS+, open bar). Values are expressed as a percent of tissue values in control broilers and calculated from values reported by Bottje et al.¹¹. An asterisk (*) indicates values were different from control ($P<.05$).

C. Compromised antioxidant capacity and lipid peroxidation in PHS

Based on the histological evidence of inflammation in many tissues of birds with PHS¹², we hypothesized that antioxidant levels would be lower in broilers having PHS. To test this hypothesis¹⁰, birds were either housed under control or low ventilation conditions to induce PHS. A compromised antioxidant capacity was demonstrated since measured antioxidants (tocopherol, ascorbate, and GSH) levels were generally lower than controls at any age in lung and liver of broilers with fulminant symptoms of PHS (PHS+) (Figure 4). Uric acid concentrations were lower in the lung and liver of PHS+ birds than Controls at 3 weeks whereas higher urate levels in lung and plasma of PHS+ broilers at 5 weeks could be a consequence of an hypoxic-induced increase in xanthine oxidase activity as outlined previously. Higher levels of K^+ and lower levels of Cl^- in the serum of PHS+ birds could be indicative of oxidative damage to membranes or Na^+/K^+ ATPase activity^{84,85}. Although part of the tissue antioxidant depression could be due to lower feed intake typically observed with PHS, the results of this study¹⁰ indicate that a compromised antioxidant status may indeed be involved in the etiology of PHS.

Because tissue tocopherol was depressed before ascorbate or GSH¹⁰ (Figure 4), a second experiment was designed to determine if vitamin E supplementation

would improve antioxidant capacity, decrease lipid peroxidation, and lower PHS mortality¹¹. To test this hypothesis, broilers were maintained under low ventilation conditions and implanted with either; a) a time-release vitamin E pellet (VE), b) a placebo pellet (PL), or c) were left intact (non-implanted, NI). Broilers maintained under normal ventilation conditions were designated as controls. PHS mortality was elevated in the PL and NI groups of birds whereas the vitamin E implant lowered PHS mortality to levels that were not different from controls (Figure 5). A higher numerical incidence of PHS in the VE implant group compared to the other groups determined by necropsy between 5 and 6 weeks suggests that early supplementation of vitamin E may have delayed the development of PHS in birds that would normally have succumbed much earlier to the disease. VE implanted birds also exhibited hematocrits, right ventricular weight ratios and plasma lipid peroxides that were not different from controls.

Dale and Villacres¹³¹ did not observe a decrease in ascites mortality with increased levels of vitamin E in the diet. This experiment, conducted at altitude (8,100 ft) with a mixed run (males and females) of broilers, reported only the incidence of PHS mortality. Possibly, differences due to increased dietary vitamin E might have been observed if only males had been used which have faster growth rates and higher incidence of PHS mortality.

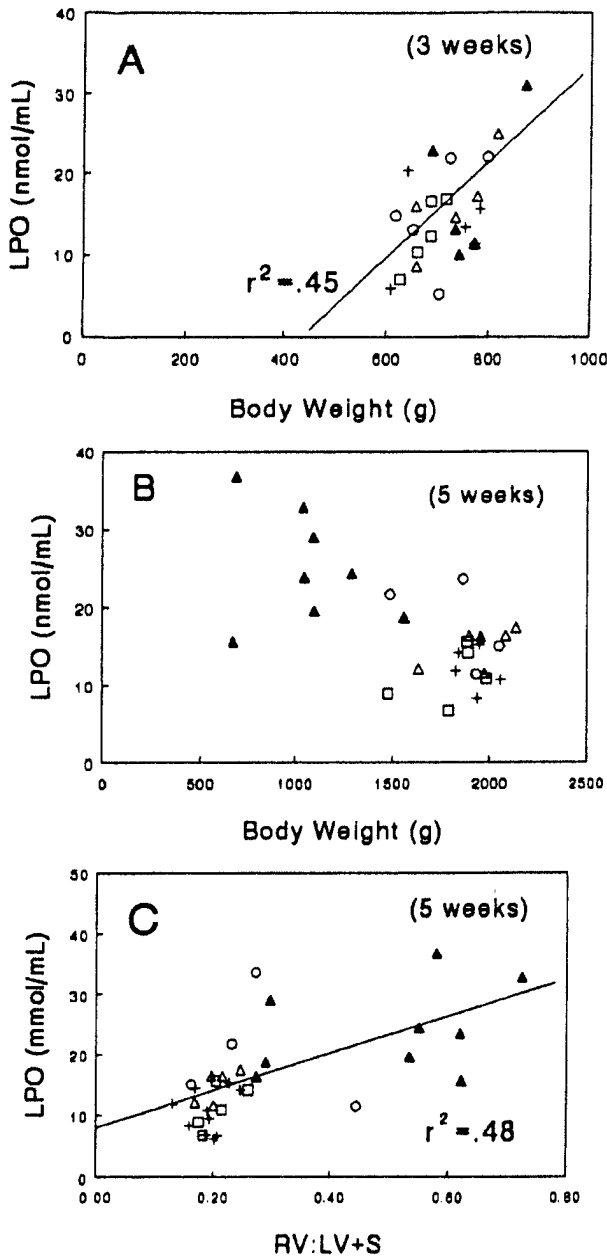


Fig. 7. Relationship between plasma lipid peroxide (LPO) concentrations and body weight in broilers at 3 (A) and 5 (B) weeks of age, and between plasma LPO and the ratio of right ventricle to left ventricle plus septum (RV/LV+S) as an indicator of pulmonary hypertension in broilers at 5 weeks of age. Values are represented from broilers maintained under control ventilation conditions (+CON) from those maintained under low ventilation conditions that were received a vitamin E implant (\square VE), a placebo implant (\circ PL), no implant (Δ NI) or which had fulminant pulmonary hypertension syndrome (\blacktriangle PHS+). Significant (P05) correlation coefficients are shown in between parameters are designated in A and C. The figure is reprinted from Bottje et al.¹¹ with permission from the Poultry Science Association.

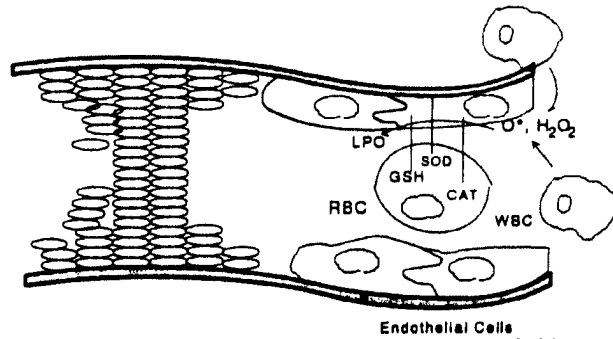


Fig. 8. A summary of antioxidant protection provided by erythrocytes.

Birds provided higher dietary levels of vitamin E might have exhibited an attenuation of PHS symptomology as was observed in our study¹¹, however, indicators of PHS such as right ventricular hypertrophy, vascular congestion, elevated hematocrit, and ascites fluid accumulation were not investigated in this study¹³¹.

Figure 6 presents data for vitamin E and GSH values in the liver and lung expressed as percent of control of VE, PL, NI and PHS+ broilers. As in the previous study¹⁰, liver and lung tocopherol levels in PHS+ were lower in comparison to controls, although the difference was not significant in the lungs at 3 weeks of age. The most dramatic effect of the vitamin E implant on tissue antioxidants was on lung α -tocopherol levels. At 3 and 5 weeks of age, α -tocopherol concentrations in the lung of broilers receiving the vitamin E implant were 199% and 168%, respectively, of control values. As the lung, specifically the pulmonary vasculature, seems to be the primary organ involved in the development of PHS, and thus particularly vulnerable to oxidative stress, the elevation in α -tocopherol combined with the lower PHS mortality suggests that pulmonary antioxidant protection may be particularly important in lowering PHS mortality. The importance of vitamin E in lower oxidative stress in lungs was recently demonstrated by Hybertson *et al.* (1995)¹³². In their experiment, it was shown that providing a vitamin E aerosol directly to the lung of rats in which acute oxidative injury produced by neutrophil infiltration of lung following interleukin 1 treatment, elevated lung tocopherol and markedly attenuated oxidative damage to the lung. Liver α -tocopherol values were also elevated in the VE implant group compared to controls, but were not different from values in the PL group at 3 and 5 weeks (Figure 6). A sparing of hepatic GSH could be observed in the VE group as hepatic GSH was 136% of controls and significantly greater than values in the other groups at 3 weeks of age. Thus, the vitamin

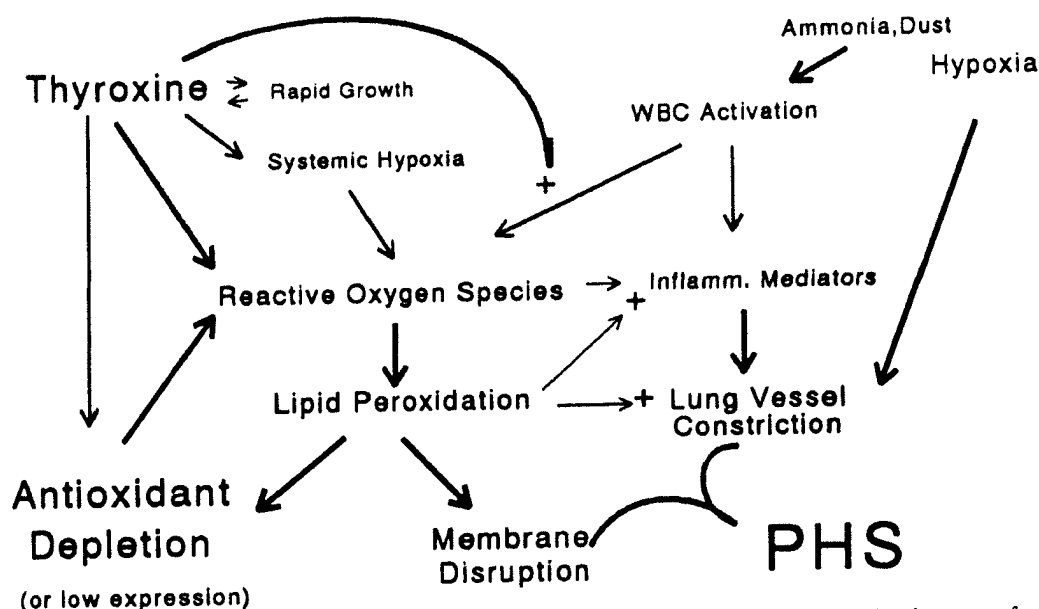


Fig. 9. A summary of events discussed in the text that could lead to lipid peroxidation and the development of pulmonary hypertension syndrome

E implant not only enhanced pulmonary antioxidant capacity, but also enhanced hepatic antioxidant capability at 3 weeks of age by increasing both α -tocopherol and GSH concentrations.

Plasma lipid peroxide values were significantly elevated in PHS+ broilers at 5 weeks¹¹. Interestingly, there was a significant linear relationship between body weight and plasma lipid peroxides at 3 weeks of age (Figure 7A), indicating that faster growing birds had higher plasma lipid peroxides than did slower growing ones, while at five weeks of age, it was mainly birds with PHS that exhibited elevated levels of plasma lipid peroxides (Figure 7B). The association between rapid growth and increased PHS mortality has been recognized for many years. The data in Figure 7A and 7B provide implicit evidence that fast growth may be associated with higher rates of lipid peroxidation and therefore higher rates of free radical and ROS production. There was also a direct correlation between plasma lipid peroxides and the right ventricle to left ventricular weight ratio which is used as an index of pulmonary hypertension (Figure 7C). These results provide further evidence of lipid peroxidation and antioxidant involvement in the etiology of PHS.

By lowering or preventing lipid peroxidation, the beneficial effect of vitamin E on lowering PHS mortality may have been accomplished indirectly by preventing peroxide-induced inhibition of prostacyclin synthesis in the lung. Prostacyclin synthetase is inhibited by high levels of lipid peroxides^{92,93,133,134}. Vasodilation of

pulmonary vasculature may be critical in preventing PHS as inclusion of compounds in the diet that promote pulmonary vasodilation effectively lowered PHS mortality^{135,136}. Adequate vitamin E levels in the lung may be crucial for maintaining prostacyclin synthase activity¹³³ as vitamin E deficiency has shown to decrease prostacyclin formation^{137,138}. Thus, the protective mechanistic role of vitamin in lowering PHS mortality may have a direct antioxidant component as well as an indirect role in maintaining vasodilatory prostacyclin production.

Other interesting observations in this experiment¹¹ included those regarding erythrocyte antioxidants. Erythrocyte antioxidants protect other tissues, particularly lung, against tissue damage produced by reactive oxygen species¹³⁹. In fully functional, healthy erythrocytes, significant oxidative damage is prevented by a very efficient antioxidant system, consisting of a number of antioxidant compounds and enzymes¹⁴⁰ as illustrated in Figure 8. While there were no differences in SOD or catalase activity among the groups at three and five weeks of age, there was a general decrease in catalase activity in all groups at five weeks compared to values at three weeks of age. A decrease in erythrocyte catalase activity could be hypothesized to contribute to an increased susceptibility to oxidative stress in older birds and thus, indirectly to the development of PHS. Birds that exhibited the most severe symptoms of PHS (*i.e.* elevated hematocrit, right ventricle to left ventricle plus septum

ratio, and lowest body weights), exhibited the lowest erythrocyte catalase activity.

Besides exhibiting elevated levels of plasma lipid peroxides, birds with PHS also exhibited lower levels of erythrocyte, liver and lung GSSG. Low levels of GSSG in these tissues could be symptomatic of general systemic oxidative stress. Under conditions of oxidative stress in which reducing equivalents required for GSH peroxidase activity become limiting, GSSG is actively transported out of erythrocytes⁸¹ and other cells⁸² in an effort to maintain redox state and to prevent oxidation or protein disulfide formation. The lower levels of GSSG in erythrocytes, liver, and lung obtained from birds with PHS, combined with elevated plasma lipid peroxides indicates that birds with fulminant PHS were experiencing considerable oxidative stress.

Another factor that could predispose broilers to oxidative stress and the development of PHS may be inherently low GSH levels in the liver. Overall GSH metabolism may be considerably different in birds as several reports have observed hepatic GSH in avian species to be 25-50% lower than in mammals^{10,11,141-149}. Furthermore, mitochondrial GSH values in broilers may be as much as 80% lower than typically reported values in mammals (unpublished observations). Low GSH concentrations would make avian mitochondria particularly susceptible to lipid peroxidation during oxidative stress. Low concentrations of GSH in the liver could be significant since the liver synthesizes and exports GSH into the circulation for uptake and utilization by other tissues¹⁵⁰. GSH values in other tissues (kidney, heart, lung and brain) were comparable to mammalian tissue GSH concentrations¹⁴⁸. Kelly *et al.* 1992ab^{146,147}, however, reported renal GSH levels in broilers that were 25 to 100% higher than has been observed in the mammalian kidney.

The reason for lower levels of hepatic GSH in avian species is not apparent but might be attributed to one or more of the following postulated mechanisms. Thyroxine is one of several hormones known to stimulate sinusoidal export of GSH from the liver¹⁵¹. Thus, increased thyroid hormone secretion necessary to sustain the high levels of growth in broilers, might continually stimulate GSH export into the general circulation. Another possible explanation for low hepatic GSH levels in broilers is its utilization for feather and muscle development. Two potentially very large GSH sinks in a rapidly growing bird are represented by muscle and feathers which require high amounts of cysteine to support protein deposition and keratin synthesis, respectively. Murphy and King¹⁴¹ hypothesized that GSH is released from the liver in molting sparrows during the dark cycle to support feather and muscle growth during periods when the bird was not

feeding. The results of their studies demonstrated that GSH was released from both the liver and muscle to support continued feather synthesis during the dark cycle and that hepatic GSH buffered against large diurnal fluctuations in free cysteine pools and ultimately large changes in protein synthesis^{141,152}. Thus, the lower hepatic GSH levels in younger broilers^{10,11} could also be due to an increased demand of GSH for muscle or feather growth as demonstrated by Murphy and King^{141,152}. There are probably other explanations for low levels of hepatic GSH in broilers but regardless of the mechanism, low hepatic GSH may cause broilers to be particularly susceptible to oxidative stress.

It is possible that the success of the vitamin E implant in lowering PHS mortality¹¹, while Dale and Villacres¹³¹ observed no effect of increased dietary vitamin E, may be partially attributed to timing of vitamin E supplementation. It has long been recognized that an increase in dietary vitamin E intake does not immediately coincide with an increase in tissue levels of vitamin E; 7 to 10 days may be required before increases in tissue levels of vitamin E are observed. Thus, providing a high level of vitamin E at one day of age to broiler chicks may have been responsible for the observed antioxidant protection against PHS. An increased need for antioxidants during the initial few weeks of broiler growth may stem from the fact that birds have an explosive period of lung development in late-prehatch and early post-hatch^{97,153,154}. It is reasonable to hypothesize that a severe lag in lung development relative to rapid body growth may place birds in a temporary period of relative hypoxia. Furthermore, as hypoxia has been shown to be a potent stimulus for free radical production, antioxidant supplementation during the very early growth period may be essential in preventing free radical generation and the advent of metabolic diseases such as PHS.

5 SUMMARY

Based on the arguments presented above, the overall biochemical and physiological events leading to ascites might be simply summarized as follows: a primary tissue hypoxia stemming from inadequate pulmonary capacity and/or metabolic rates in excess of pulmonary capacity, followed by a secondary generation of reactive species leading to tissue damage which further accentuates ROS generation. The ROS generated in this manner might be likened to spiralling hyperthermia associated with heat stress mortality. In this case however, oxygen, rather than temperature, is the critical component requiring adjustment or regulation.

Figure 9 is an attempt to illustrate the physiological and biochemical events leading to lipid peroxidation and

onset of PHS that have been described above. With pulmonary insufficiency, systemic hypoxia develops. PHS was first observed at high altitudes as a result of high altitude hypoxia. Systemic hypoxia is a powerful stimulus for the body to increase red blood cell production to increase oxygen carrying capacity of the blood. The increase in red blood cell numbers will improve oxygen carrying capacity, but a potential negative consequence is that any free iron released into the blood; e.g. by peroxidative damage erythrocyte membranes, could increase hydroxyl radical formation in the presence of hydrogen peroxide. Lung damage from release of iron has been demonstrated by Seibert *et al.*¹⁵⁵.

Cellular hypoxia is accentuated by the high metabolic demands associated with rapid growth that occurs in broilers. The high rate of metabolism and release of waste products such as carbon dioxide and hydrogen ion, dilate precapillary arterioles, leading to an increase in cardiac output. Increased cardiac output will only further magnify pulmonary hypertension. With the virtually constant feeding that occurs in broiler operations, the high metabolic demand placed by ingestion of feed and hepatic metabolism could result in very low oxygen concentration at the tissue level. Reactive oxygen species generated in the intestines would be sent to the liver via the portal vein. In addition, if an inflammatory response is triggered by dust and ammonia, by cells dying from lack of oxygen, then lipid peroxidation could be occurring in many different tissues simultaneously. As peroxidation continues in systemic tissue, lipid peroxide levels in the blood returning to the heart and lungs will rise. The elevated peroxide levels could damage cells lining blood vessels in the heart and lungs (causing a shift from endothelium dependent relaxation to endothelium dependent vasoconstriction). Increased peroxidation in the lungs can cause vasoconstriction directly, or indirectly through stimulation of local vasoconstricting hormones (leukotrienes, thromboxane, inhibition of PGI₂).

A second problem related to cardio-pulmonary insufficiency in broilers are indications that mitochondrial function is compromised by hypoxia (e.g. ref. 128). Thus, systemic hypoxia would cause cellular hypoxia and increased free radical production in mitochondria and other organelles, raising the likelihood that lipid peroxidation would occur. Release of chemotactic hydroperoxides would cause phagocyte and macrophage to infiltrate tissues which in turn release additional chemotactic factors such as cytokines with subsequent infiltration of heterophils. Once in the tissues, the phagocytes would release more free radicals and reactive compounds, further accentuating tissue damage. As indicated above, inflammation is an integral part of the pathophysiology of PHS^{10,112}.

The end product of all these biochemical events could be a) direct damage and constriction of lung vasculature, b) potential damage to heart tissues, and c) increased demand for cardiac output. All of these factors would simply magnify the problems in birds predisposed to PHS. Possibly, broilers with inadequate mitochondrial antioxidant protection fall into the category of ones that are PHS susceptible with fast growth but with only marginal or moderate lung development⁹⁵.

Recent reports by Wideman *et al.*^{135,136} indicated that pulmonary vasodilators (furosemide and L-arginine) decreased PHS-induced mortality. As shown in Figure 8, besides lowering pulmonary vascular pressure, pulmonary vasodilators would increase tissue oxygenation in broilers thereby blocking the chain of events that lead to hypoxia driven free radical production and lipid peroxidation. The effect of pulmonary vasodilators on lipid peroxidation and tissue antioxidants during the development of PHS warrants further investigation. Dietary combinations of pulmonary vasodilators and antioxidants such as vitamin E might prove an effective solution to lowering PHS-induced mortality.

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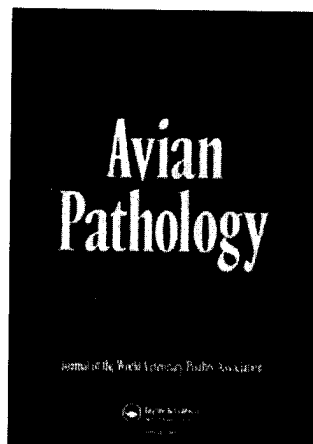
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Prophylactic action of lipoic acid on oxidative stress and growth performance in broilers at risk of developing ascites syndrome

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The objective of this study was to assess the effects of dietary supplementation with lipoic acid (LA) on broilers maintained at 2235 m above sea level with high risk to develop ascites syndrome (AS). A total of 2040 chicks were fed under commercial conditions with water and specific diets *ad libitum* during 7 weeks in two consecutive experiments. Mortality and indicators of performance and oxidative stress were compared weekly in broilers fed a basal diet plus 0, 10, 20, or 40 parts/10⁶ LA. The effects of LA at 40 parts/10⁶ were also studied during the initial 3 weeks or the last 4 weeks of the production cycle. Diets supplemented with 40 parts/10⁶ of LA during 7 weeks significantly improved feed conversion, decreased general mortality and mortality attributable to AS, and lowered thiobarbituric acid reactive substances and hydroxyl radicals in liver, and increased total glutathione pool. Smaller doses or shorter periods of exposure to LA were partially effective. In conclusion, LA under our experimental conditions has a prophylactic action in broilers with high risk to develop AS due to oxygen availability limitation.

Introduction

Ascites syndrome (AS) is well characterized from the epidemiological, clinical, and anatomopathological points of view. Accumulative evidence favors the idea that decreased oxygen tension or increased oxygen requirements causes AS in broiler chickens (Decuyper *et al.*, 2000). If both conditions are present, risk of developing AS increases as more oxygen is required to sustain a high feed conversion rate and rapid growth, and oxygen tension decreases at altitudes > 2000 m above sea level, as occurs on farms near Mexico City where mortality rates attributable to AS in this region can be as high as 29% (Arce *et al.*, 1990). Thus, hypoxic conditions in tissues may result in a vicious circle in which reduction in pulmonary oxygen tension constricts arterioles, causing an increase in lung

arterial pressure; this in turn results in right ventricle hypertrophy and gradual reduction in right ventricle output, which substantially increases venous pressure of the hepatic veins that results in edema in the lungs, the abdominal cavity, and the hydropericardium (Julian, 1993; Decuyper *et al.*, 2000).

However, Bottje & Wideman (1995) and our research group (Díaz-Cruz *et al.*, 1996) have shown that oxidative stress—that is, an important imbalance between production of reactive oxygen species (ROS) and reactive nitrogen species on the one hand, and antioxidant defenses on the other—is included in development of AS. To date, experiments are insufficient to define whether this oxidative stress is a cause or a consequence in the pathogenesis of AS. In any event, temporary reduction in feed intake (Suárez & Rubio, 1989)

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that in other systems limits ROS (Sohal & Wein-druch, 1966), alternative lighting schedules (Buys *et al.*, 1998), and dietary supplementation with anti-oxidants and free radical scavengers, as detailed in the Discussion of this work, have been attempted to decrease AS mortality.

The purpose of the study reported here was to evaluate whether dietary supplementation with lipoic acid (LA) would be a better alternative than the use of other prophylactic measures to ameliorate oxidative stress, growth performance, and cumulative mortality attributable to naturally developing AS in broiler flocks maintained under commercial conditions at an altitude of 2235 m above sea level (i.e. at high risk to develop AS). Indicators of oxidative stress in this work included thiobarbituric acid reactive substances (TBARS), total glutathione (TG), the presence of the hydroxyl radical (OH^\bullet), and the electrophoretic mobility of catalase, which is modified by the presence of singlet oxygen and gives rise to conformers with increased electrophoretic mobility (Lledias & Hansberg, 2000). Antioxidant properties of lipoic acid are well known, its use has some advantages over antioxidant vitamins, and its pharmacologic properties have been summarized and reported earlier (Packer *et al.*, 1995; Biewenga *et al.*, 1997).

Materials and Methods

Experiment 1

Young (1-day-old) broilers (1032 chicks; Avian Farm x Arbor Acres strain) were used in this study. Broilers were weighed and randomly assigned to four dietary treatment groups (258 birds/group). Birds were housed for the duration of the 7-week study in six pens (43 birds/pen) per treatment at an altitude of approximately 2235 m above sea level. The chicks were brooded at 32°C during week 1; thereafter, the temperature was reduced gradually each week to reach 24°C at day 28. All chicks were fed the same starter diet (1–21 days) containing 3000 kcal/kg metabolizable energy (ME) and a finisher diet (22–49 days) with 3100 kcal/kg ME, both diets formulated to meet or exceed all requirements of the National Research Council (1994). Birds were randomly assigned to the control group or groups with LA. Control birds were fed the basal diet without additional LA, while birds with LA were fed the basal diet to which LA (10, 20, and 40 parts/ 10^6) was added.

Thiobarbituric acid reactive substances. The concentration of TBARS was assessed by the method of Zentella de Piña *et al.* (1993). Briefly, homogenates of hepatic tissues were filtered through a cheesecloth, and an aliquot of the filtered homogenate was incubated with 0.1 ml of 0.15 M phosphate buffer (pH 7.4) for 30 min at 37°C. Then, 1.5 ml of 20% acetic acid (pH 2.5) and 1.5 ml of 0.8% thiobarbituric acid were added. The mixture was placed in boiling water for 45 min. Tubes were allowed to cool, and 1 ml of 2% KCl and 5 ml butanol:pyridine (15:1) solution was added to each tube. Tubes were mixed vigorously and the absorbance of the organic layer was measured by a spectrophotometer set at 532 nm. The concentration of TBARS in the samples was calculated with an extinction coefficient of $1.56 \times 10^5 \text{ M/cm}$ (Wills, 1969). The protein concentration was determined by the method of Bradford (1976).

Total glutathione. The concentration of TG was determined by the enzymatic method of Akerboom & Sies (1981). Briefly, 1.0 ml of 0.1 M buffer phosphate, 100 μl sample containing 0.5 to 2 n moles of

glutathione, 20 μl nicotinamide adenine dinucleotide phosphate reduced (NADPH), 20 μl 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and 20 μl glutathione reductase were placed in cuvette. After mixing the contents of the cuvette, the increase in absorbance at 412 nm was recorded. A blank assay without glutathione was run separately. For calibration, the procedure was repeated using oxidized glutathione instead of the sample. The temperature was controlled at 25°C.

Experiment 2

Broilers of 1 day old (1008 chicks, Ross x Ross strain) were fed the same starter and finisher ration as described in experiment 1 except for the ME (starter ration, 3100 kcal/kg; finisher ration, 3200 kcal/kg). Broilers were weighed and randomly assigned to four dietary treatment groups and were housed under the same conditions as in experiment 1. The dietary treatment groups were as follows; control basal diet group without LA throughout study; with LA (40 parts/ 10^6) in basal diet for 1–21 days and with LA (40 parts/ 10^6) in basal diet days for 22–49 only, and with LA (40 parts/ 10^6) in basal diet throughout study.

In both experiments, feed and water were provide *ad libitum*. Feed consumption and body weight were recorded weekly. Liver samples were obtain weekly after euthanizing the birds by cervical dislocation; the liver was removed, blotted dry, and desiccated. Subsequently, the amounts of TBARS, TG, and OH^\bullet in samples were determined. Birds were included in mortality by ascitic syndrome if they died with ascites without abdominal fluid accumulation but with obvious ascitic syndrome symptoms including right ventricular dilation, hydropericardium, and vascular congestion.

Concentration of hydroxyl radical. The concentration of hydroxyl radical (OH^\bullet) was assessed using the method described by Nash (1953). Briefly, 1 g hepatic tissue was homogenized in 5 ml of 20% trichloroacetic acid; an aliquot (500 μl) of homogenate was mixed in 1 ml Krebs–Ringer solution added with 0.1 mM FeCl_3 , 0.2 mM ethylenediamine at pH 7.4 to avoid superoxide formation and 33 mM dimethylsulfoxide as the substrate for hydroxyl radicals in production of formaldehyde (Hallinan *et al.*, 1991). Hydroxyl free radicals were measured as changes in absorbance at 412 nm in the supernate by the Hantzsch reaction in which the formaldehyde formed, as an index of OH^\bullet free radicals, was calculated using an extinction coefficient of $800 \times 10^3 \text{ M/nm}$.

Catalase. Catalase activity in polyacrylamide gels was analyzed by the method of Lledias & Hansberg (2000). Briefly, homogenates of liver tissues (1 g wet weight) were incubated with 5 ml of 20 mM 4-(2-hydroxy)-1-piperazineethanesulfonic acid (pH 7.2) containing 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 0.1 mM deferriferrioxamine B mesylate (Desferal). Samples of 18 μg protein in 10 μl were loaded into each lane. Gels were run at 150 V for 2.5 h; immediately after electrophoresis, these were stained for catalase activity, detected by incubating the gel for 5 min in 5% methanol and then rinsing three times with tap water for 10 min in 10 mM H_2O_2 . The gel rinsed with tap water was incubated in a 1:1 mixture of freshly prepared 2% potassium ferric cyanide and 2% ferric chloride. A blue color developed in the gel except at zones in which H_2O_2 was decomposed by catalase.

Statistical analysis. One-way analysis of variance was used to compare differences in variables among and within experimental groups using statistical analysis calculated with SAS software (SAS Institute, 1995) with means comparisons using the Tukey test. A probability level of $P < 0.05$ was considered statistically significant.

Results

Indicators of productivity

Data on feed consumption, growth rate, and feed conversion together with information on overall mortality and mortality attributable to AS are considered in this work as indicators of productiv-

ity. These indicators of productivity are reported first for experiment 1; that is, broilers fed with diets having lower ME and distributed in four experimental groups (without LA, and with three different levels of LA added to the diet). For better analysis of results, these were organized in two stages. The initial stage included the first 21 days of treatment with a diet having an ME of 3000 kcal/kg diet, while the final stage included days 22 to 49 of treatment with a diet having an ME of 3100 kcal/kg diet. Additionally, results of the entire 49-day study are presented (Table 1). During the initial stage, a statistically significant difference was found for general mortality by comparing number of birds fed with control diet with those fed with LA-enriched diet (10 parts/10⁶) (Table 1). Greater differences appeared in indicators of productivity of broilers during the final stage. Inclusion of LA in at least one diet resulted in the following statistically significant differences: decrease in feed consumption at 10, 20, and 40 parts/10⁶; better growth performance at 40 parts/10⁶; better feed conversion at 20 and 40 parts/10⁶; lower general mortality at 40 parts/10⁶, and much lower mortality attributable to AS at 40 parts/10⁶ (Table 1). For the entire experimental period, only feed conversion at 20 and 40 parts/10⁶, general mortality at 40 parts/10⁶, and mortality attributable to AS at 10, 20, 40 parts/10⁶ were improved by addition of LA to diets (Table 1). The effect (i.e. LA decreasing mortality attributable to AS) was increased as higher doses of antioxidant compounds were added to the diet (Table 1).

Beneficial results due to LA-enhanced diet were evident during 22 to 49 days in experiment 1 as compared with scarce results reached during the initial stage of study (Table 1). Hence, a second experiment was carried out in which supplementation of LA (40 parts/10⁶) was offered to broilers in one or both stages. Control groups included broilers with an LA-free diet. To establish a higher risk in broilers to develop AS spontaneously, the ME content of diets was increased to 3100 kcal/kg during initial stages and to 3200 kcal/kg during the final stage. Additionally, a different chick strain was tested to validate LA preventing AS due to limitation in oxygen availability. Results with the same indicators of productivity as those presented in Table 1 are presented in Table 2 for the second experiment. An increase in the ME content of diets augmented feed consumption, growth performance, general mortality, and mortality attributable to AS, and decreased feed conversion in all groups (except one) of birds receiving 40 parts/10⁶ LA (comparison of data in Table 1 versus Table 2). Broilers included in the second experiment receiving diets with increased ME were subjected to a higher challenge to develop AS, as confirmed by an increase in mortality attributable to AS (see Table 2 versus Table 1). Despite this, supplementation with 40 parts/10⁶ LA during the entire 49 days of the study reproduced the benefits recorded for LA in the first experiment; that is, a significant increase in growth performance and decrease in both feed conversion and mortality attributable to AS (Table 2). It is of interest that the main effect of LA in

Table 1. Indicators of productivity in broilers fed in stage 1 with a diet having a metabolizable energy content of 3000 kcal/kg and fed in stage 2 with a diet having a metabolizable energy content of 3100 kcal/kg: effect of lipoic acid

Variable	Age (days)	Diets supplemented with lipoic acid			
		0 (control diet)	10 parts/10 ⁶	20 parts/10 ⁶	40 parts/10 ⁶
Feed consumption (g)	1 to 21	858 ± 14 ^A	858 ± 12 ^A	863 ± 37 ^A	841 ± 46 ^A
	22 to 49	3751 ± 50 ^A	3450 ± 30 ^B	3387 ± 40 ^C	3438 ± 40 ^B
	1 to 49	4609 ± 21 ^A	4308 ± 15 ^A	4250 ± 48 ^A	4379 ± 33 ^A
Growth performance (g body weight)	1 to 21	584 ± 20 ^A	585 ± 20 ^A	582 ± 40 ^A	568 ± 50 ^A
	22 to 49	1783 ± 60 ^A	1678 ± 70 ^A	1725 ± 50 ^A	1832 ± 50 ^B
	1 to 49	2367 ± 57 ^A	2262 ± 30 ^A	2308 ± 18 ^A	2400 ± 58 ^A
Feed conversion (g feed/g body weight gained)	1 to 21	1.46 ± 0.02 ^A	1.46 ± 0.03 ^A	1.48 ± 0.07 ^A	1.48 ± 0.08 ^A
	22 to 49	2.10 ± 0.08 ^A	2.05 ± 0.07 ^A	1.96 ± 0.08 ^B	1.87 ± 0.06 ^C
	1 to 49	1.95 ± 0.02 ^A	1.89 ± 0.02 ^A	1.84 ± 0.01 ^B	1.82 ± 0.02 ^B
General mortality (%)	1 to 21	3.52 ± 0.81 ^A	0.78 ± 0.03 ^B	1.17 ± 0.07 ^{AB}	1.93 ± 0.08 ^{AB}
	22 to 49	5.64 ± 0.99 ^A	4.30 ± 1.14 ^{AB}	3.51 ± 1.03 ^{AB}	1.57 ± 0.49 ^B
	1 to 49	9.16 ± 1.43 ^A	5.08 ± 1.16 ^{AB}	4.68 ± 1.08 ^{AB}	3.50 ± 0.93 ^B
Mortality attributable to ascites syndrome (%)	1 to 21	0.76 ± 0.48 ^A	0.00 ± 0.00 ^A	0.00 ± 0.00 ^A	0.38 ± 0.39 ^A
	22 to 49	3.92 ± 0.88 ^A	2.31 ± 0.84 ^{AB}	1.96 ± 0.74 ^{AB}	0.76 ± 0.48 ^B
	1 to 49	4.68 ± 0.71 ^A	2.31 ± 0.38 ^B	1.96 ± 0.77 ^B	1.14 ± 0.38 ^B

Values presented as mean (± standard error of the mean). Within each row, values with different superscript letters differ significantly ($P < 0.05$).

Table 2. Indicators of productivity in broilers fed in stage 1 with a diet having a metabolizable energy content of 3100 kcal/kg and fed in stage 2 with a diet having a metabolizable energy content of 3200 kcal/kg: effect of lipoic acid, 40 parts/10⁶, during different experimental periods

Variable	Age (days)	Days on which 40 parts/10 ⁶ lipoic acid was included in the diet			
		Day 0 (control diet)	Days 1 to 21	Days 22 to 49	Days 1 to 49
Feed consumption (g)	1 to 21	949 ± 6.97 ^A	924 ± 11.78 ^A	944 ± 9.85 ^A	923 ± 8.14 ^A
	22 to 49	4109 ± 12.27 ^A	4041 ± 27.20 ^A	4029 ± 24.03 ^A	4044 ± 40.68 ^A
	1 to 49	5058 ± 12.85 ^A	4966 ± 22.02 ^A	4970 ± 30.64 ^A	4967 ± 24.51 ^A
Growth performance (g body weight)	1 to 21	649 ± 5.45 ^A	655 ± 2.25 ^A	659 ± 3.51 ^A	664 ± 4.75 ^A
	22 to 49	2160 ± 27.34 ^A	2205 ± 25.75 ^{AB}	2219 ± 21.38 ^{AB}	2269 ± 26.65 ^B
	1 to 49	2809 ± 17.77 ^A	2873 ± 19.82 ^{AB}	2877 ± 24.56 ^{AB}	2933 ± 25.70 ^B
Feed conversion (g feed/g body weight gained)	1 to 21	1.46 ± 0.02 ^A	1.41 ± 0.02 ^{AB}	1.43 ± 0.02 ^{AB}	1.39 ± 0.01 ^B
	22 to 49	1.90 ± 0.01 ^A	1.83 ± 0.02 ^B	1.82 ± 0.02 ^B	1.78 ± 0.01 ^B
	1 to 49	1.80 ± 0.01 ^A	1.73 ± 0.01 ^B	1.73 ± 0.01 ^B	1.69 ± 0.01 ^B
General mortality (%)	1 to 21	4.37 ± 2.16 ^A	3.57 ± 1.46 ^A	1.59 ± 0.48 ^A	3.57 ± 0.53 ^A
	22 to 49	12.69 ± 1.70 ^A	11.11 ± 2.00 ^A	11.90 ± 1.87 ^A	9.52 ± 1.94 ^A
	1 to 49	17.06 ± 2.97 ^A	14.68 ± 2.16 ^A	16.66 ± 1.62 ^A	13.09 ± 2.01 ^A
Mortality attributable to ascites syndrome (%)	1 to 21	0.40 ± 0.39	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	22 to 49	9.12 ± 1.67 ^A	5.55 ± 1.00 ^{AB}	6.74 ± 1.29 ^{AB}	3.96 ± 1.00 ^B
	1 to 49	9.52 ± 1.62 ^A	5.55 ± 1.00 ^{AB}	6.74 ± 1.29 ^{AB}	3.96 ± 1.00 ^B

Values presented as mean (± standard error of the mean). Within each row, values with different superscript letters differ significantly ($P < 0.05$).

preventing AS was reproduced with a different chick strain.

Markers of oxidative stress

For the first experiment, the TBARS and TG content in hepatic tissues of broilers euthanized weekly throughout the study were measured. Dietary supplementation with three increasing doses of LA resulted in significantly lower TBARS values from days 21 to 42 with a single exception; that is, day 35 in birds supplemented with 10 parts/10⁶ LA compared with values in the control group (Table 3 and Figure 1). Although there were no differences in hepatic TG during the first 14 days of the experiment in the four groups, higher hepatic TG values were observed later in broilers fed with supplemented diets as doses of LA were increased (Table 4 and Figure 2). Using 40 parts/10⁶ LA diet, an increase in TG as compared with the control diet was statistically significant from days 21 to 35.

In the second experiment, indicators of ROS were monitored measuring OH[•] radicals and electrophoretic mobility of catalase in liver. Results included in Figure 3 show the value results of OH[•] recorded in birds receiving LA during the first 21 days of the experiment; the content of hepatic OH[•] remained reduced despite the fact that the supplement was withdrawn in these animals during days 22 to 49. In the group receiving LA from days 22 to 49, values of OH[•] during the first stage were similar to control values: once LA was added to the diet beginning on day 22, the hepatic value of OH[•] was statistically lower than in the liver of broilers with a control-fed diet except at day 49

(Figure 3). Changes in the electrophoretic mobility of catalase were not observed in liver samples of broilers whether fed with or without LA; that is, no evidence of singlet oxygen generation was observed (results not shown).

Discussion

The main objective of our research work was to prevent the incidence of AS in broilers maintained under strict commercial conditions at 2235 m above sea level. Our reasoning was that a 10% decrease in atmospheric oxygen availability at an altitude of 2235 m above sea level is sufficient to lower oxygen tension in the inspired air of broilers; this, together with increased oxygen requirements to sustain rapid growth rates and high feed efficiencies, created hypoxic conditions in tissues and favored onset of pulmonary hypertension syndrome, whose ultimate consequence is AS (Decuyper *et al.*, 2000). Hypoxic conditions also facilitated progressive establishment of oxidative stress, which played an important role in genesis of tissue damage (McCord, 1985).

The incidence of AS has been experimentally enhanced by decreasing ventilation and temperature in environmental chambers (Enkvetchakul *et al.*, 1993) or by dietary administration of 1.5 parts/10⁶ triiodothyronine (Decuyper *et al.*, 1994). Furthermore, in broiler flocks maintained at an altitude of 2235 m the risk to develop AS increased (Arce *et al.*, 1990; Díaz-Cruz *et al.*, 1996). In these cases, cumulative mortality attributable to AS was near 30% and evidence of tissue oxidative stress as

Table 3. Mean (\pm standard error of the mean) values of thiobarbituric acid reactive substances (nmol/mg protein) in liver of broilers being fed a basal diet supplemented with lipoic acid

Age (days)	Lipoic acid			
	0 (control)	10 parts/ 10^6	20 parts/ 10^6	40 parts/ 10^6
1	0.416 \pm 0.208 ^A	0.316 \pm 0.168 ^A	0.229 \pm 0.155 ^A	0.338 \pm 0.168 ^A
7	0.168 \pm 0.037 ^A	0.082 \pm 0.035 ^A	0.051 \pm 0.048 ^A	0.093 \pm 0.066 ^A
14	0.384 \pm 0.140 ^A	0.195 \pm 0.097 ^A	0.172 \pm 0.068 ^A	0.211 \pm 0.092 ^A
21	0.939 \pm 0.244 ^A	0.467 \pm 0.103 ^B	0.353 \pm 0.850 ^B	0.336 \pm 0.134 ^B
28	0.897 \pm 0.137 ^A	0.235 \pm 0.085 ^C	0.521 \pm 0.114 ^B	0.294 \pm 0.095 ^C
35	0.650 \pm 0.182 ^A	0.579 \pm 0.097 ^A	0.285 \pm 0.111 ^B	0.307 \pm 0.178 ^B
42	0.487 \pm 0.102 ^A	0.206 \pm 0.045 ^B	0.196 \pm 0.056 ^B	0.186 \pm 0.088 ^B

Means with different superscript letters differ significantly ($P < 0.05$, $n = 6$) against the control.

described later was documented in at least two of these studies. In general, the lung and liver concentrations of ascorbic acid, tocopherol, and TG were lower in birds maintained in the low

ventilation chamber compared with control birds (Enkvetchakul *et al.*, 1993). In the model of spontaneous AS by high altitude, TBARS values in the liver and heart were 2.4-fold and 2.8-fold

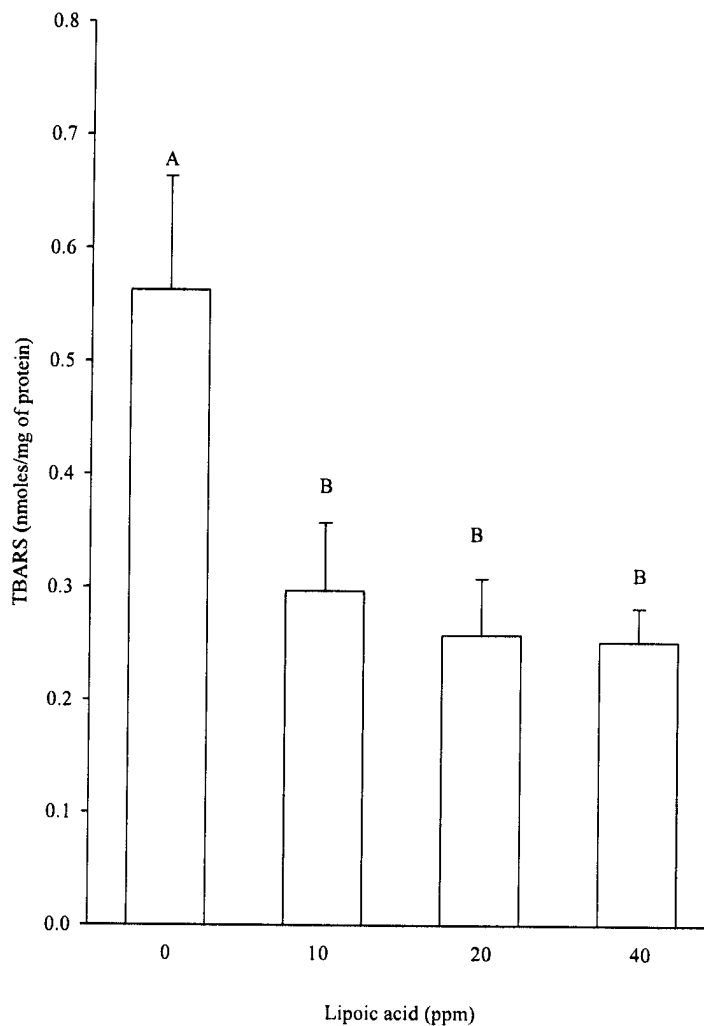


Figure 1. Summary of thiobarbituric acid reactive substances (TBARS) \pm standard error of the mean recorded in liver samples from broilers being fed with a basal diet without lipoic acid (LA), or this basal diet supplemented with LA, during 42 days. Each bar represents the mathematical sum of each value of each column in Table 3 divided by 7; that is, the number of weekly experiments (each with six birds) performed throughout the 42 days. Means with different letters differ significantly ($P < 0.05$) against the control.

Table 4. Means (\pm standard error of the mean) values of total glutathione ($\mu\text{mol/g ww}$) in liver of broilers being fed a basal diet supplemented with lipoic acid

Age (days)	Lipoic acid			
	0 (control)	10 parts/ 10^6	20 parts/ 10^6	40 parts/ 10^6
1	2.40 ± 0.66^A	2.53 ± 0.47^A	3.40 ± 0.63^A	2.33 ± 0.55^A
7	2.16 ± 0.41^A	2.78 ± 0.31^A	2.56 ± 0.33^A	3.22 ± 0.79^A
14	2.40 ± 0.72^A	2.73 ± 0.21^A	2.06 ± 0.46^A	3.07 ± 1.10^A
21	1.32 ± 0.39^B	1.00 ± 0.26^B	2.19 ± 0.58^A	2.93 ± 0.43^A
28	2.03 ± 0.38^B	1.04 ± 0.58^C	2.66 ± 0.42^B	3.32 ± 0.40^A
35	0.89 ± 0.59^B	3.22 ± 0.53^C	2.01 ± 0.42^A	2.54 ± 0.46^A
42	2.07 ± 0.26^A	2.50 ± 0.62^{AB}	3.19 ± 0.71^B	2.48 ± 0.39^{AB}

Means with different superscript letters differ significantly ($P < 0.05$, $n = 6$) against the control.

higher in birds with clinical signs of AS compared with control birds, respectively (Díaz-Cruz *et al.*, 1996).

Although temporary reduction in feed intake (Suárez & Rubio, 1989) and alternative lighting schedules (Buys *et al.*, 1998) have proved successful in reducing the incidence of AS, the search for alternative possibilities including generalized use of feed supplemented with specific anti-oxidants con-

tinues. Diets supplemented with α -tocopherol acetate exerted a dose-dependent response on hepatic and pulmonary α -tocopherol concentrations but had no effect on growth performance or mortality attributable to pulmonary hypertension syndrome provoked by reducing ventilation and decreasing temperature (Bottje *et al.*, 1995). In the model of high risk of AS at an altitude of 2235 m, vitamin E-supplemented diets resulted in better growth per-

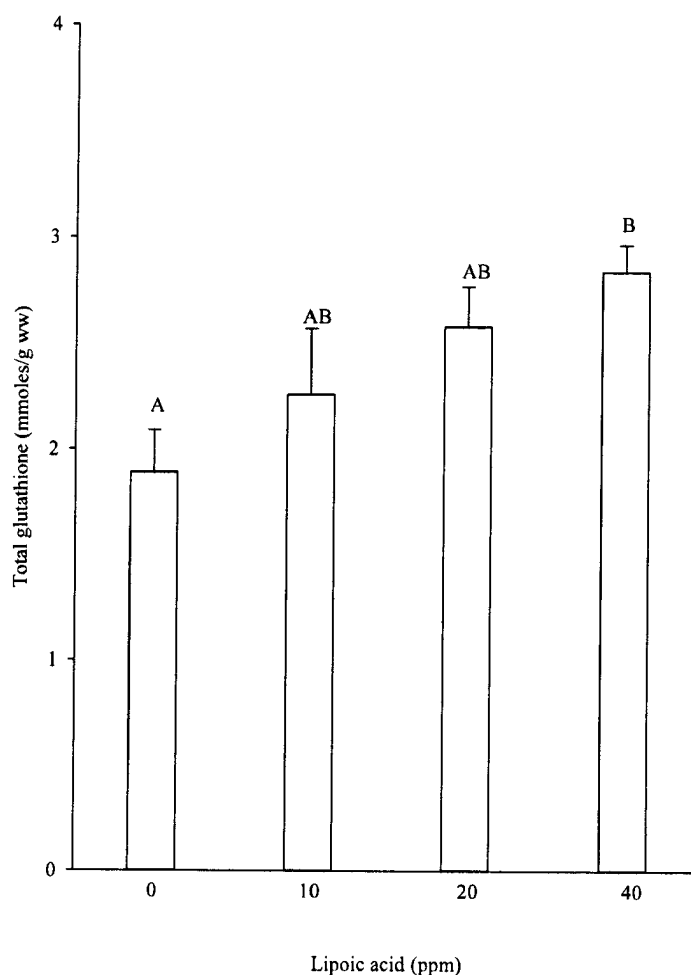


Figure 2. Total glutathione \pm standard error of the mean recorded in liver samples from broilers being fed with a basal diet without lipoic acid (LA), or this basal diet supplemented with LA during 42 days. Each bar represents the mathematical sum of each value of each column in Table 4 divided by 7; that is, the number of weekly experiments (each with six birds) performed throughout the 42 days.

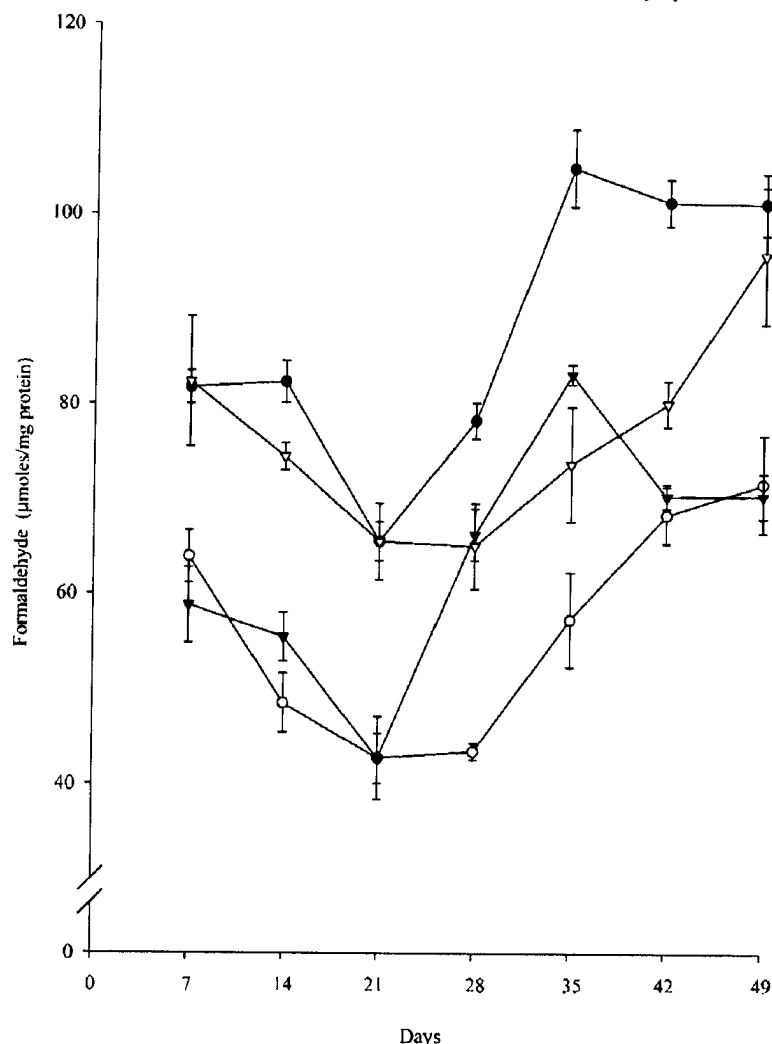


Figure 3. Mean \pm standard error of the mean values for OH^\cdot free radical pool in liver tissues obtained every 7 days from broilers fed with the following diets: basal diet without lipoic acid (LA) (solid circle); with 40 parts/10⁶ LA during 49 days (open circle); with 40 parts/10⁶ LA during the first 21 days of the experiment (solid triangle); or with 40 parts/10⁶ LA on days 22 to 49 of the experiment (open triangle). The assay was performed with four birds in each group at each time point.

formance, lower rates of feed conversion, and lower TBARS content; nonetheless, the vitamin did not modify mortality attributable to AS (Villar-Patiño *et al.*, 2002).

Administration of triiodothyronine (T_3) to broilers increased susceptibility for AS; in this model, food supplementation with vitamin C significantly reduced ascites mortality while displaying no effect on performance parameters (Hassanzadeh *et al.*, 1997). In broilers maintained at 2235 m altitude, vitamin C-supplemented diets resulted in significantly lower feed consumption and lower rates of feed conversion; nevertheless, supplementation had no effect on mortality attributable to AS (Villar-Patiño *et al.*, 2002). In the same model in birds grown at an altitude of 2235 m, diets supplemented with piroxicam, a non-steroidal anti-inflammatory drug that prevented hepatic increase of triacylglycerols and TBARS as well as decrease in TG levels resulting from acute ethanol intoxication in rats

(Zentella de Piña *et al.*, 1992), lowered the TBARS pool in the lung, liver, and heart of broilers with high risk to develop AS. However, the compound did not change the growth rate, feed consumption, nor feed conversion and did not modify cumulative mortality caused by AS (Valle *et al.*, 2001).

In this work, further information reinforced findings that (1) oxidative stress was the molecular basis of AS and that (2) supplementation of diets with LA improved molecular indicators of oxidative stress, performance indexes, and cumulative mortality attributable to AS. LA (40 parts/10⁶) administered to broiler flocks maintained under commercial conditions at 2235 m altitude resulted in a lower TBARS content, a lower pool of OH^\cdot radicals, and higher levels in TG (Figures 1–3) in liver of birds with high risk to develop AS. Supplementation also improved feed consumption, feed conversion, growth performance and, above all, decreased cumulative mortality attributable to

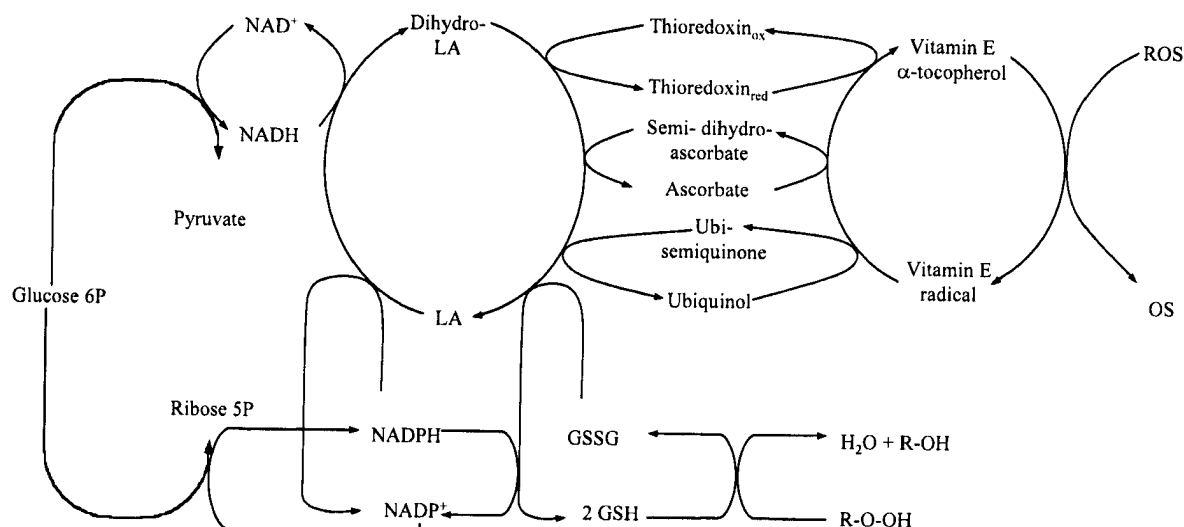


Figure 4. Pivotal role of lipoic acid (LA), which used reduced coenzymes generated by cytosolic glucose oxidation to recycle oxidized antioxidants. The reaction of an antioxidant (vitamin E, vitamin C, reduced glutathione (GSH)) and a reactive oxygen species (ROS) (or H_2O_2) eliminates ROS (or H_2O_2), but the antioxidant is converted into a product no longer able to function. This oxidized product is regenerated to its native form to function again via the dehydro LA/LA redox couple. OS, oxygen species; GSSG, oxidized glutathione; NAD, nicotinamide adenine dinucleotide (oxidized); NADH, nicotinamide adenine dinucleotide (reduced); NADP, nicotinamide adenine dinucleotide phosphate (oxidized); NADPH, nicotinamide adenine dinucleotide phosphate (reduced).

AS (Tables 1 and 2). These results were observed in two different chick strains, even after increasing dietary ME to levels higher than in average diets commonly used at this altitude, which would increase broiler acceptability to develop AS. To the best of our knowledge, diet supplementation with LA is advantageous over other supplementation to prevent AS, at least in the model in which birds were maintained at high altitude.

Figure 4 includes a possible mechanism (modified from Biewenga *et al.* (1997) and Lodge & Packer (2000)) to explain the beneficial action of LA in decreasing oxidative stress previous to onset of AS. Cytosolic oxidation of suitable substrates (glucose) generates dehydrolipoate from feed lipoate; dehydrolipoate has a central role in regenerating reduced glutathione (GSH), reduced thioredoxine, ascorbate, and ubiquinol; the former two regenerate α -tocopherol. Some of these reduced regenerated compounds are useful to inactivate ROS and other pro-oxidant molecules such as H_2O_2 , and in this way decrease cellular oxidative stress.

Acknowledgements

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RÉSUMÉ

Action prophylactique de l'acide lipoïque sur le stress oxydatif et sur les performances de croissance des poulets de chair qui présentent un risque élevé de développer un syndrome ascite

L'objectif de cette étude a été d'évaluer les effets d'une supplémentation de l'aliment avec de l'acide lipoïque (LA) chez des poulets élevés à 2,235 m d'altitude qui présentent un risque élevé de développer un syndrome ascite (AS). Au cours de deux essais successifs, deux mille quarante poulets de chair ont été nourris dans des conditions commerciales avec de l'eau et différents régimes *ad libitum* durant 7 semaines.

La mortalité, les indicateurs de performances ainsi que le stress oxydatif ont été comparés toutes les semaines chez des poulets de chair nourris avec un régime de base auquel a été ajouté 0, 10, 20 ou 40 ppm de LA. Les effets de LA à 40 ppm ont été également étudiés durant les

3 premières semaines ou les 4 dernières semaines du cycle de production. Les régimes supplémentés avec 40 ppm de LA durant 7 semaines ont amélioré l'indice de conversion, diminué la mortalité générale et la mortalité spécifique attribuée à l'AS, diminué les substances réactives d'acide thiobarbiturique et les radicaux OH au niveau du foie et augmenté le glutathion total.

Des régimes avec du LA administré à plus petites doses ou durant des périodes plus courtes, ont été partiellement efficaces. En conclusion, le LA dans nos conditions expérimentales a eu une action prophylactique chez les poulets de chair qui présentaient un risque élevé de développer un AS dû à une quantité limitée d'oxygène disponible.

ZUSAMMENFASSUNG

Prophylaktische Wirkung von Liponsäure (DL-1,2-Dithiolan-3-Valeriansäure) auf oxidativen Stress und Mastleistung in Broilern mit Risiko zur Entwicklung des Aszitisyndroms

Ziel dieser Studie war es, die Effekte der diätetischen Supplementierung von Liponsäure (LA) auf Broiler zu untersuchen, die auf 2235 Metern über dem Meeresspiegel mit erhöhtem Risiko, das Aszitisyndrom (AS) zu entwickeln, gehalten wurden. Insgesamt wurden 2040 Hühnerküken in zwei aufeinanderfolgenden Experimenten unter kommerziellen Bedingungen sieben Wochen lang mit Wasser und speziellem Futter *ad libitum* versorgt. Bei Broilern, die mit einem Grundfuttermittel supplementiert mit 0, 10, 20 oder 40 ppm LA gefüttert wurden, wurden Mortalität, Leistungsdaten und Indikatoren für oxidativen Stress wöchentlich verglichen. Der Einfluss von 40 ppm LA wurde außerdem in den ersten drei oder letzten vier Wochen des Produktionszyklus untersucht. Das mit 40 ppm supplementierte Futter über 7 Wochen verabreicht verbesserte die Futterverwertung und verringerte die allgemeine und die AS bedingte Mortalität, reduzierte Thiobarbitursäure-reaktive Substanzen sowie OH⁻ Radikale in der Leber und erhöhte den totalen Glutathion-Pool signifikant. Geringere Dosen oder kürzere Supplementierungsphasen mit LA waren teilweise wirksam. Daraus kann geschlossen werden, dass LA unter experimentellen Bedingungen eine prophylaktische Wirkung auf Broiler hat, die aufgrund einer limitierten Sauerstoffverfügbarkeit ein erhöhtes Risiko besitzen an AS zu erkranken.

RESUMEN

Acción profiláctica del ácido lipoico sobre el estrés oxidativo y el crecimiento en pollos de engorde con riesgo de desarrollar el síndrome ascítico

El objetivo de este estudio fue el de valorar los efectos de la suplementación en dieta con ácido lipoico (LA) en pollos de engorde mantenidos a 2,235 m por encima del nivel del mar con alto riesgo de desarrollar síndrome ascítico (AS). Un total de 2,040 pollos fueron alimentados bajo condiciones comerciales con agua y dietas específicas *ad libitum* durante 7 semanas en dos experimentos consecutivos. La mortalidad y los indicadores del crecimiento y del estrés oxidativo se compararon semanalmente en pollos de engorde alimentados con dietas basales más 0, 10, 20 o 40 ppm de LA. Los efectos del LA a 40 ppm también fueron estudiados durante las primeras tres semanas o las últimas cuatro semanas del ciclo de producción. Las dietas suplementadas con 40 ppm de LA durante 7 semanas mejoraron significativamente el índice de conversión, disminuyeron la mortalidad general y la mortalidad atribuible a AS, y disminuyeron las sustancias reactivas de ácido tiobarbitúrico y los radicales OH⁻ en el hígado e incrementaron el *pool* total de glutatión. Los tratamientos con dosis menores o periodos de exposición mas cortos a LA fueron parcialmente efectivos. En conclusión, el LA tiene acciones profilácticas bajo condiciones experimentales en pollos de engorde que presenten riesgo elevado de desarrollar AS debido a la limitación en el aporte de oxígeno.



Nonesterified fatty acids and endothelial dysfunction

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Abstract

Nonesterified fatty acids (NEFA) induce vascular effects, such as the inhibition of insulin-induced nitric oxide (NO) production. In small arteries, relaxation is largely NO-independent. We aimed to assess the effect of elevated NEFA on NO-independent vasodilation. Protocols were performed before and after 120 min of a lipid emulsion infusion. We performed: (1) the flow-induced dilatation of the brachial artery (NO-dependent), (2) intrabrachial bradykinin (BK) infusion before and after inhibition of prostaglandins (PG) and NO and (3) intraarterial infusion of ouabain, a Na^+/K^+ ATPase blocker, alone or in combination with BaCl_2 , a potassium channel blocker. No changes in flow-mediated vasodilation were induced by elevated NEFA. After the inhibition of PG and NO, BK increased forearm blood flow (FBF) similarly, indicating that the increase in FBF is not dependent on NO and PG production. However, elevated NEFA blunt FBF on both occasions. Coinfusion of ouabain with BaCl_2 caused a significant decline in FBF in baseline condition ($-48 \pm 5\%$, $p < 0.01$). This effect on FBF was blunted at high NEFA ($-28 \pm 2\%$, $p < 0.01$ vs. baseline condition). In conclusion, elevated NEFA do not impair NO-dependent vasodilation; this effect appears to be mediated by a reduced potassium-mediated vasodilation. This makes more likely their negative action on metabolism and haemodynamic coupling.

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Keywords: Nitric oxide; Endothelial-derived hyperpolarizing factor; Free fatty acids

1. Introduction

Elevations of plasma nonesterified fatty acids (NEFA) inhibit insulin-stimulated glucose uptake both acutely and chronically [1–3].

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In the recent years, it has also been shown that NEFA induce important vascular effects [4,5]. In vivo, high NEFA induce a reduced leg blood flow response to metacholine infusion, a reduced forearm blood flow to acetylcholine infusion and an impaired insulin-mediated vasodilation [6–8]. Thus, the NEFA-induced inhibition of nitric oxide (NO) production can be considered as an alternative effect by which these compounds interfere with intermediary metabolism. These effects may also explain the findings of the Paris Prospective Study, which showed that NEFA is a predictive risk factor for sudden death [9].

Studies have shown heterogeneous vascular relaxation in vessels of different sizes; in small arteries, there is NO-independent, rather than NO-dependent, relaxation [10,11]. Thus, in term of metabolic/haemodynamic coupling, NO-independent appears more relevant than the NO-dependent vasorelaxation. The aim of this study was to examine the role of short-term elevation of NEFA on NO-independent relaxation in the vascular bed of the human forearm.

2. Material and methods

Fourteen normal nonsmoking healthy volunteers (4 females and 10 males), 25 ± 3 years old and with a BMI of $22.5 \pm 1.6 \text{ kg/m}^2$ (means \pm S.D.), with no risk factors for coronary artery disease, participated after giving informed consent. The study protocol was approved by the local Ethical Committee, and each subject gave his/her written informed consent. NEFA were determined at the beginning and at the end of each study. All subjects were studied on three separate occasions. All studies were performed in a fasting state in a temperature-controlled room in baseline conditions, and the after 120 min of infusion of a lipid emulsion (Intralipid 10%), which was given as a bolus of 0.15 g/kg body weight, followed by continuous infusion of 2 ml min^{-1} plus heparin ($40 \text{ U kg}^{-1} \text{ h}^{-1}$).

2.1. Study A

The endothelium-dependent, flow-induced dilatation of the left brachial artery was assessed (NO dependent). This was measured noninvasively by high-resolution ultrasound Doppler technique as described [12]. The diameters of three consecutive cardiac cycles were averaged. After baseline measurements were recorded, a standard adult-size blood pressure cuff was placed 1–2 cm below the ipsilateral antecubital fossa and inflated at 200 mmHg for 5 min and then released. Flow-mediated vasodilation was determined as the percent diameter change of the postocclusion measurement relative to the mean of three baseline measurements.

2.2. Study B

Bradykinin (BK) was intrabrachially infused at three different steps (50, 100 and 200 ng 100 ml forearm volume (FAV) $^{-1} \text{ min}^{-1}$), before and after inhibition of prostaglandins and NO, with indomethacin and N^G -mono-L-methyl-arginine (L-NMMA) [13]. The same responses to BK were repeated in baseline condition, in the presence of NO-clamp

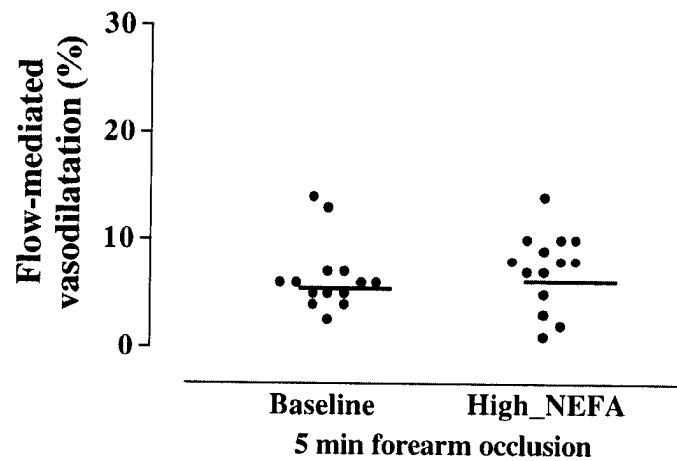


Fig. 1. Flow-mediated vasodilatation of the brachial artery after 5-min occlusion in baseline condition (bottom) and at high NEFA (top) for each subject. Bars represent the mean values.

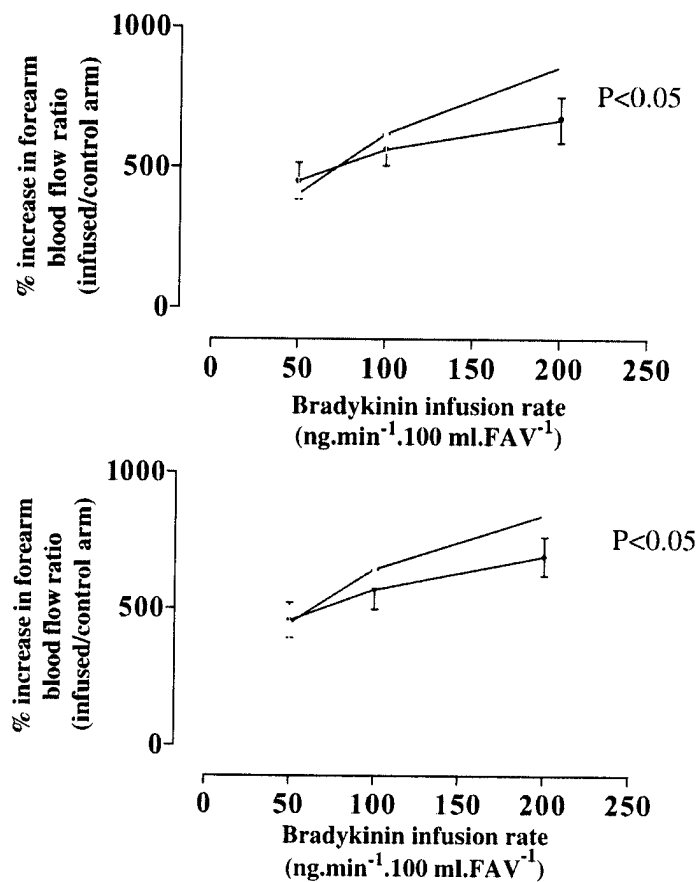


Fig. 2. Percentage increase in forearm blood flow ratio (infused/control arm) during intrabrachial infusion of bradykinin at three different doses in baseline condition (grey squares) and at high NEFA (black circles) before (top) and during the NO-clamp (bottom). The significance refers to the differences between the two experimental conditions at the highest rate of infusion. Data are means \pm S.E.M.

(i.e. during restoration of basal blood flow after inhibition of endogenous NO by L-NMMA, obtained by SNP infusion). Forearm blood flow (FBF) was measured by plethysmography after hand circulation was excluded. Blood flow measurements were performed in the contralateral (control) forearm as well, to exclude systemic effects. Blood pressure was recorded immediately before each measurement with a noninvasive technique.

2.3. Study C

To test the hypothesis that K^+ may act as EDHF, we used the approach proposed by Dawes et al. [14]. The effects of either the intraarterial infusion of ouabain alone (for 9 min), a Na^+/K^+ ATPase blocker, or in combination with $BaCl_2$ (for 4 min), a rectifying potassium channel (K_{IR}) blocker, on basal FBF was assessed.

2.4. Calculations and statistical analysis

Results are expressed as means \pm S.E.M. Statistical analysis was performed with two-way ANOVA for repeated measures. Statistical significance was taken at the 5% level ($p < 0.05$).

3. Results

In response to the combined lipid emulsion/heparin infusion, there was a threefold increase in plasma FFA from 460 ± 54 to $1278 \pm 135 \mu\text{mol/l}$ ($p < 0.001$).

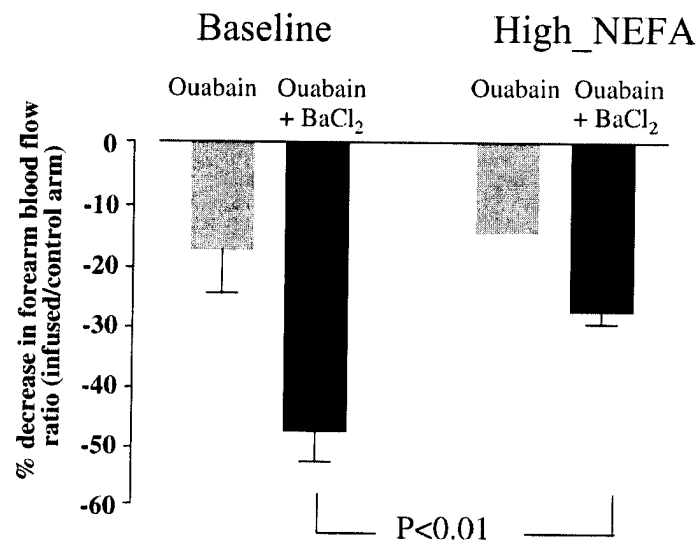


Fig. 3. Percentage change in resting FBF during brachial artery infusion of ouabain alone (black bars) or in combination with $BaCl_2$ (grey bars). Data are means \pm S.E.M.

In study A, no changes in flow-mediated vasodilation were induced by elevated NEFA ($6.5 \pm 0.9\%$ in the baseline conditions and $7.3 \pm 0.9\%$ during high NEFA; $p = \text{n.s.}$) (Fig. 1).

Cumulative doses of BK increased FBF by $381 \pm 76\%$, $623 \pm 100\%$ and $848 \pm 104\%$, respectively, for 50, 100 and 200 ng 100 ml FAV⁻¹ min⁻¹. During NO clamping, BK increased FBF in a similar manner (by $446 \pm 65\%$, $645 \pm 67\%$ and $844 \pm 61\%$, respectively), indicating that the increase in blood flow is not dependent on NO and PG production (Fig. 2). At high NEFA, without and with NO clamp, there was a blunted response of forearm blood flow in response to BK. Ouabain infusion (Fig. 3) caused a slight decrease ($-18 \pm 7\%$, $p < 0.05$) in FBF in baseline condition: similar effect was observed during high NEFA ($-15 \pm 5\%$). On the contrary, the coinfusion of ouabain with BaCl₂ caused a significant decline in FBF in baseline condition ($-48 \pm 5\%$, $p < 0.01$). However, this inhibitory effect on FBF was significantly blunted in the presence of high NEFA ($-28 \pm 2\%$, $p < 0.01$ vs. baseline condition).

4. Discussion

This study shows that acutely elevated NEFA impairs the NO-independent endothelial vasodilation. This effect appears to be mediated by a reduced potassium-mediated vasodilation. Furthermore, we were unable to observe postischemic changes in the diameter of the brachial artery above basal, which is a measure of endothelial integrity of this conduit artery [15]. This approach determines the endothelial function in a large conduit artery, where the relaxation is almost entirely NO-dependent [16]. At variance, Lundman et al. [17] showed that the same triglyceride emulsion, maintained for 1 h, results in decreased vasoreactivity of the brachial artery as measured by noninvasive high-resolution ultrasound technique. Unlike NO, the role of EDHF in mediating relaxation is enhanced with decreasing vessel size [10,11]: the lack of a significant effect of elevated NEFA on brachial artery would support the hypothesis that these substrates might exert a negative action on endothelial EDHF rather than on NO production. To prove this, we administered BK before and during the so-called NO clamp [13]. This approach suggests that the increased NEFA concentration negatively acts on NO-independent vasodilatory mechanism(s). However, the exact identification of an EDHF remains to be an extremely contentious point. For this reason, a functional definition of EDHF has been proposed: agonist-induced, endothelium-dependent relaxations that are not blocked by inhibitors of NO synthase or cyclooxygenase but are inhibited by potassium channel blockers [18]. To further clarify this issue, we performed study C, in which we infused inhibitors of both K_{IR} and Na⁺/K⁺ ATPase, as previously suggested [14]. This approach allowed us to demonstrate that the NEFA-mediated inhibition on NO-independent vasodilation is largely mediated by a blunted potassium-dependent vasodilation. As shown in Fig. 3, while the inhibition of Na⁺/K⁺ ATPase plays a little, if any, effect, the infusion of BaCl₂ elicited a lower decrease in baseline FBF in the presence of high NEFA. Since BaCl₂ selectively blocks inwardly rectifying K⁺ channels [19], with this approach, we were able to demonstrate that NEFA blunt the

NO-independent vasodilatory response, which is mainly mediated by a decreased K^+ -induced vasorelaxation.

In conclusion, acutely elevated NEFA concentration does not alter postischemic flow-mediated vasodilation. By adopting the NO clamp technique, we showed that the increase of these substrates selectively impairs NO-independent vasodilation. Moreover, the decrease in FBF mediated by inhibition of K_{IR} is significantly blunted by high NEFA levels. Therefore, these substrates interfere with the K^+ -induced vasorelaxation. Since this vasorelaxation is more pronounced at small vessel levels, this makes more likely their negative action on metabolism and haemodynamic coupling.

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REVIEW

Non-esterified fatty acids and blood pressure elevation: a mechanism for hypertension in subjects with obesity/insulin resistance?

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The prevalence of hypertension in individuals with obesity or type II diabetes is substantially elevated. Increased levels of non-esterified fatty acids (NEFAs) in abdominally obese subjects were reported to contribute in the development of various disturbances related to the metabolic syndrome, such as hepatic and peripheral insulin resistance (IR), dyslipidaemia, β -cell apoptosis, endothelial dysfunction and others. However, the involvement of NEFAs in the development of hypertension has been much less studied in comparison to other mechanisms linking IR and central obesity with blood pressure (BP) elevation. This article reviews the existing evidence on the relation between NEFA and hypertension in an attempt to shed a light on it. *In vivo* data from both animal

and human studies support that acute plasma NEFA elevation leads to increase in BP levels, whereas epidemiological evidence suggests a link between increased NEFA levels and hypertension. Further, accumulating data indicate the existence of several pathways through which NEFAs could promote BP elevation, that is α_1 -adrenergic stimulation, endothelial dysfunction, increase in oxidant stress, stimulation of vascular cell's growth and others. The above data support a possible important role of NEFA in hypertension development in patients with obesity and the metabolic syndrome and raise hypotheses for future research.

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Keywords: non-esterified fatty acids; hypertension; obesity; insulin resistance; type II diabetes

Introduction

In the initial modern descriptions of the insulin resistance (IR) or metabolic syndrome, IR was proposed to be the primary disorder, causally related to the development of the rest disturbances, that is impaired glucose tolerance or type II diabetes, hypertension and hypertriglyceridaemia.^{1,2} However, central obesity, a factor promptly added to the above cluster,^{2,3} was soon found to be connected in a more complex way with IR (i.e. to be not a consequence but rather a cause of IR), as well as to play important roles in the development of the rest disorders of the syndrome.⁴

One of the most important factors connecting obesity with the development of related metabolic abnormalities is the elevated release of free or non-esterified fatty acids (NEFAs) from abdominal

adipocytes in patients with central obesity, which results in increase in plasma NEFA concentration and turnover.^{5,6} NEFAs have been shown to induce both hepatic and peripheral IR,^{7,8} to activate apoptotic pathways in pancreatic β cells,⁹ to promote endothelial dysfunction¹⁰ and to increase the production of plasminogen activator inhibitor-1.¹¹ Most importantly, increased NEFA supply of the liver, primarily owing to the incomplete insulin-mediated suppression of lipolysis in this type of subjects, is the initial step for the development of the characteristic lipid disorders of the metabolic syndrome.¹²

With regard to hypertension, several studies have clearly shown a causal association of IR and compensatory hyperinsulinaemia with blood pressure (BP) elevation, through mechanisms like sodium retention, stimulation of the sympathetic nervous system, promotion of vascular cell's growth or impairment of insulin-mediated vasodilatation in insulin-resistant states.¹³ On the other hand, central obesity is also known to induce the development of hypertension through increased activity of adipose tissue renin–angiotensin–aldosterone (RAAS), sympathetic activation and other mechanisms, usually in close connection with IR.^{14,15}

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NEFA elevation has been previously implicated to be one of the latter mechanisms linking abdominal obesity to BP elevation.^{15,16} However, the research interest in this mechanism has never been particularly intense, despite the accumulation of a considerable amount of data supporting a possible relevant role for NEFAs. Therefore, the aim of this review was to summarize the existing evidence on the direct effects of NEFA elevation on BP, as well as possible pathophysiologic mechanisms connecting NEFAs with BP increase, in an attempt to shed an up-to-date light on this association and to raise hypotheses for future research.

Search strategy

A systematic literature search of MEDLINE/PubMed and EMBASE databases was performed to identify English-language articles published from 1966 until May 2006 that reported data on the association between NEFA and hypertension. Search terms used were 'non-esterified fatty acids', 'free fatty acids', 'NEFA', 'FFA' in combination with 'blood pressure' and 'hypertension'. Reference lists of identified articles were also evaluated for additional relevant papers and information. Articles providing information on the effect of NEFAs on BP levels or on

possible mechanisms linking NEFA with the development of hypertension were included.

The effect of NEFAs on BP levels

The first indications on the association of NEFAs with BP elevation came from an *in vivo* study in minipigs¹⁷ (Table 1). In this study, Intravenous infusions of Intralipid (a triglyceride emulsion containing primarily glycerol esters of two NEFAs, oleic and linoleic acid) and heparin (to activate endothelial lipoprotein lipase, which in turn, hydrolyses the ester bond and releases NEFA in the circulation)^{16,18} were performed, in order to increase circulating NEFA levels. This NEFA elevation was associated with a significant increase in peripheral vascular resistance and a rise in BP of approximately 30 mm Hg.¹⁷

Subsequently, Grekin *et al.* observed that infusion of sodium oleic (which derives from combination of oleic acid with sodium) in portal or femoral veins of rats resulted in acute increases in mean BP of 29 and 13 mm Hg, respectively. Moreover, chronic (1 week) administration of sodium oleic was associated with an elevation of 16 mm Hg in mean BP vs baseline.¹⁹ Portal infusion of sodium oleic also resulted in an increase in plasma catecholamines, suggesting that

Table 1 Studies reporting direct effects of plasma NEFA elevation on BP levels

Study	Animal model/type of human subjects	Method to produce NEFA elevation	Main findings
Bulow <i>et al.</i> ¹⁷ Grekin <i>et al.</i> ¹⁹	Gottingen minipigs Sprague-Dawley rats	Acute i.v. infusion of Intralipid and heparin (a) Acute and (b) chronic (1 week) i.v. infusion of sodium oleic	Increase in MBP of 30 mm Hg (a) Increase in MBP of 29 and 13 mm Hg after infusion in portal and femoral veins, respectively (b) Increase of 16 mm Hg in MBP
Grekin <i>et al.</i> ²⁰	Sprague-Dawley rats	(a) Acute and (b) chronic (1 week) i.v. portal infusion of oleic acid (c) Acute i.v. portal infusion of linoleic acid	(a) Increase of 12 mm Hg in MBP (b) Increase of 8 mm Hg in MBP vs control animals (c) Increase of 12 mm Hg in MBP Increases of about 3–6 mm Hg in SBP and no significant differences in DBP in all experiments in comparison to normal saline infusion
Steinberg <i>et al.</i> ²³	Lean healthy humans	(a) 2-h i.v. infusion of low or high doses of Intralipid and heparin (b) Acute somatostatin administration	
Steinberg <i>et al.</i> ²⁴ de Kreutzenberg <i>et al.</i> ³⁸	Lean healthy humans Lean healthy humans	(a) Short (2 or 4 h) and (b) long (8 h) i.v. infusions of Intralipid and heparin 2-h i.v. infusion of long- or medium-chain triglycerides and heparin	(a) No significant change in MBP (b) Increase in MBP of 10 mm Hg No significant change in MBP
Stojiljkovic <i>et al.</i> ²⁵	Lean healthy humans ^a	4-h i.v. infusion of Intralipid and heparin	Increases of 10 mm Hg in SBP and 3 mm Hg in DBP
Lopes <i>et al.</i> ²⁶	Lean healthy humans with or without FHH	4-h i.v. infusion of Intralipid and heparin	Significant increase of SBP and DBP (14/7 mm Hg) in subjects with FHH and significant increase of SBP (10 mm Hg) in those without FHH
Lopes <i>et al.</i> ²⁷	Obese hypertensive and lean normotensive humans ^b	4-h i.v. infusion of Intralipid and heparin	Increases in SBP in obese hypertensives (4–5 mm Hg) and in lean normotensives (8–13 mm Hg) and nonsignificant increases in DBP on all 3 diets

Abbreviations: BP, blood pressure; DBP, diastolic blood pressure; FHH, family history of hypertension; i.v., intravenous; MBP, mean blood pressure; NEFA, non-esterified fatty acids; SBP, systolic blood pressure.

^aSubjects were for the previous 3 weeks on a diet low in antioxidants.

^bSubjects were for the previous 4 weeks on their usual diet, a diet rich or a diet low in antioxidants.

BP elevation could be related to sympathetic activation. In another study of the same group, acute and chronic portal infusions of oleic acid, as well as acute infusion of linoleic acid were again associated with significant increases in BP.²⁰

Previous human studies provided indirect evidence in favour of an association between NEFA and the development of hypertension. Egan *et al.*²¹ observed that baseline plasma levels and turnover rate of NEFAs were increased in obese compared to lean individuals, but similar between obese hypertensive and obese normotensive subjects. However, the decrease in plasma NEFA levels after insulin administration was much smaller in obese hypertensive than obese normotensive subjects; in other words, obese hypertensives displayed resistance in insulin-mediated NEFA reduction. Another finding connecting high levels of NEFAs with hypertension was that BP levels were significantly correlated with NEFA levels and turnover rate, but not glucose disposal rate. These correlations remained significant after adjustment for fasting insulin levels, IR and other parameters.²¹

Data from the population-based Paris Prospective Study also support these associations. In almost 3000 non-diabetic, non-hypertensive men followed for 3 years, baseline NEFA elevation was a highly significant risk factor for the subsequent development of hypertension (hazard ratio of 1.58) after controlling for several known risk factors and other abnormalities of the metabolic syndrome.²²

In addition to the above data, several human studies have clearly shown that acute plasma NEFA elevation leads to increase in BP (Table 1).^{23–27} Most of these studies aimed primarily to evaluate the effect of NEFAs on other parameters (i.e. endothelial function) with complicated experiments and are described in detail below, in the sections of the possible pathways linking NEFAs with hypertension. Of particular interest, however, is a study suggesting a possible link between NEFA elevation, family history of hypertension and BP regulation. In this study, 4-h Intralipid and heparin infusions were performed in lean healthy subjects with negative or positive family history of hypertension. Although NEFAs increased similarly in both groups during the infusion, diastolic BP (DBP), mean BP (MBP) and pulse pressure increased significantly only in subjects with a family history of hypertension. Systolic BP (SBP) increased in both groups, but the increase was significantly greater in subjects with positive than those with no family history of hypertension (14 ± 2 vs 10 ± 2 mm Hg).²⁶

Based on such data, it can be speculated that in subjects that typically exhibit chronic NEFA elevation, that is those with central obesity, IR and type II diabetes,^{4–6} this NEFA increase can be an important factor promoting the development of hypertension in subjects with normal BP levels, or deteriorating BP control in patients with already elevated BP. Further, some of the above data^{21,22} support not only a close

association between NEFA and BP, but also that this relation could be independent from the degree of IR and other parameters of the metabolic syndrome. This can be another interesting possibility, but needs further examination by background and epidemiological studies. The following sections describe pathophysiologic mechanisms that can be possibly involved in a NEFA–hypertension relationship.

NEFA and α_1 -adrenergic activation

In the above-mentioned study from Grekin *et al.*,²⁰ the increase in BP following portal oleic acid infusion was blunted from prazosin, an α_1 -adrenergic antagonist, but not from an angiotensin II receptor inhibitor, findings indicating the involvement of an α_1 -adrenergic mechanism in this BP elevation (Figure 1). These observations in favour of an α_1 -adrenergic-mediated action of NEFAs are in agreement with previous findings from Egan *et al.* in humans. This group reported an increase in vascular tone and vascular responsiveness in α_1 -adrenergic stimulation in obese hypertensive patients,^{28–30} which could not be explained from resistance in the vasodilating action of insulin.³¹ Assuming that this increase is caused by NEFA elevation, the investigators studied the infusion of Intralipid and heparin in hand veins of two groups of normotensive subjects. Local NEFA increase substantially reduced the dose of phenylephrine required to produce 50% of the maximal venoconstrictor response, whereas the response to angiotensin II was not altered.³²

In a subsequent study, the involvement of α_1 - or both α_1 - and α_2 -adrenergic mechanism was examined, again by local infusion of Intralipid and heparin in normotensive individuals. The relevant increase in oleic and linoleic acid was again related to an increased vasoconstrictor response to phenylephrine, which is a non-selective α_1 - and α_2 -adrenergic agonist but not to clonidine, which is a rather selective α_2 -agonist.³³ In conjunction with the above study, elevation of plasma NEFAs after infusion of Intralipid alone or Intralipid and heparin in lean normotensive subjects similarly reduced the dose of phenylephrine required to raise MBP by 20 mm Hg.³⁴ These data suggest that raising levels of plasma NEFAs and/or triglycerides enhance α_1 -adrenoceptor-mediated venoconstriction and pressor sensitivity. Other investigators reported comparably adverse effects of NEFAs on vascular function. For example, the post-ischaemic vasodilatory response of brachial artery in healthy lean subjects was significantly reduced after transient hypertriglyceridaemia that increased plasma NEFA levels.³⁵

NEFA, nitric oxide and endothelial dysfunction

Previous studies *in vitro* suggest an interference of NEFA with nitric oxide (NO) production from

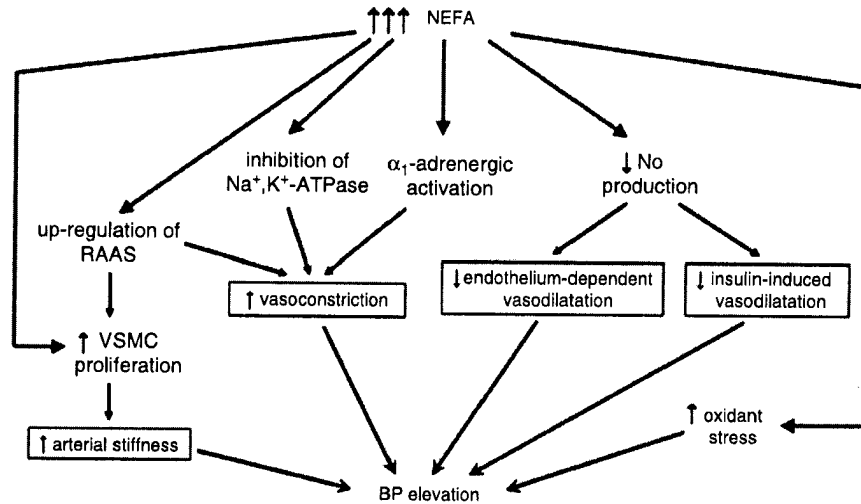


Figure 1 Possible pathways connecting elevation of plasma NEFAs with BP increase (NEFAs, non-esterified fatty acids; RAAS, renin-angiotensin-aldosterone system; VSMCs, vascular smooth muscle cells; NO, nitric oxide).

endothelial cells. Both oleic and linoleic acids were reported to cause dose-dependent reduction of endothelial NO synthase (NOS) activity in bovine pulmonary artery endothelial cells.³⁶ In another study, addition of NEFAs in endothelial cells in culture was associated with dose-dependent reductions of NO production. This reduction was also attributed to a decrease in NOS activity, as NOS concentration in endothelial cells remained unaltered.³⁷ Further, in *ex vivo* experiments in rabbit femoral artery rings oleic acid inhibited the acetylcholine-mediated vasodilatation, which is endothelium-dependent. In contrast, the endothelium-independent vasodilating action of sodium nitroprusside was not altered, a finding indicating that NEFAs were only interfering with endothelium-dependent vasodilatation.³⁶

In addition to the above findings, elegant *in vivo* experiments provided detailed information for the effects of NEFAs on endothelial function in humans. Steinberg *et al.*²³ observed that intra-arterial infusion of metacholine in femoral artery of lean healthy individuals during intravenous normal saline infusion resulted in an increase of leg blood flow up to about 300% in relation to baseline levels. After 2 h of systemic administration of either low- or high-dose Intralipid and heparin, which both increased plasma NEFA concentration, repeat of metacholine infusion caused a significantly lower increase in leg blood flow than before (maximum increase up to about 200%). Similarly, plasma NEFA elevation with the use of somatostatin, which inhibited insulin production, thus increasing lipolysis, produced a comparable disturbance of endothelium-dependent vasodilatation. In contrast, elevated NEFAs did not interfere with endothelium-independent vasodilatation, caused by sodium nitroprusside infusion. In all these experiments, during Intralipid and heparin infusion SBP measured invasively was

3–6 mm Hg higher than during the respective saline infusion, whereas DBP was not significantly different.²³ Another *in vivo* study confirmed these findings on NEFAs causing endothelial dysfunction, showing that NEFA elevation resulting from 2-h infusion of long- or medium-chain triglycerides and heparin depressed the acetylcholine-induced increase in forearm blood flow. However, in this latter study, MBP changes in all the experiments were small and insignificant.³⁸

NEFAs were also shown to interfere with basic NO production. In particular, Steinberg *et al.*²⁴ measured leg blood flow before and after infusion of *N*-monomethyl-L-arginine (L-NMMA), which is an inhibitor of endothelial NOS, in two groups of lean healthy subjects. In one group the leg blood flow response measurements were performed during saline infusion and in the other, after a 2-h infusion of Intralipid and heparin. In the group not receiving Intralipid and heparin, a decrease of leg blood flow of about 17% was observed after the L-NMMA infusion. This reduction practically reflected the contribution of NO in preservation of vascular tone under normal circumstances. However, after Intralipid and heparin administration, the L-NMMA-induced leg blood flow decrease was only 9%, which suggests that short-term NEFA elevation decreased basic NO release for about 50%.²⁴

Effects of NEFAs on insulin-mediated vasodilatation

In healthy subjects, acute administration of insulin is long known to produce endothelium-dependent vasodilatation.^{39,40} However, numerous studies have shown that this action of insulin is severely impaired in subjects with various components of the metabolic syndrome^{41,42} and this impairment

is considered a basic mechanism linking IR and compensatory hyperinsulinaemia with BP elevation.^{13,43}

In a different protocol of the above-mentioned study,²⁴ the investigators evaluated the possible involvement of NEFAs in this impairment of insulin action. Lean healthy individuals underwent two sets of euglycaemic hyperinsulinaemic clamp experiments with and without parallel increases of Intralipid and heparin. The subjects were divided into two groups, one receiving Intralipid and heparin infusion of short (2 or 4 h) and the other of long (8 h) duration. Short infusion did not produce any significant changes, but long infusion was associated with significant reductions in glucose uptake during the clamp of about 35%, in insulin-induced leg blood flow increase of 40% and in insulin-mediated NO production of 80%. In both the groups, the changes in IR during NEFA elevation was strongly correlated with the changes in insulin-mediated leg blood flow increase, which suggests a similar impact of NEFAs in the tightly coupled metabolic and vascular actions of insulin.²⁴ As far as BP is concerned, basic MBP levels were similar between the groups and did not change during the clamps performed without Intralipid and heparin infusion. Short plasma NEFA elevation did not cause significant changes in MBP either before or during the clamp experiment. However, long plasma NEFA elevation was associated with an increase of 10 mm Hg in MBP both before and during the clamp. As a result, MBP during the clamp in the group with the long NEFA elevation was significantly higher than that in the group with the short elevation.

This effect of NEFAs on insulin-mediated vasodilatation can be partly explained from their action on phosphatidylinositol 3-kinase (PI3-K), an enzyme playing a central role in insulin intracellular signalling. Both insulin-mediated glucose uptake in skeletal muscle cells and insulin-mediated NO production from endothelial cells take place through activation of PI3-K pathway.^{44,45} NEFAs have been repeatedly shown to interfere with insulin-mediated glucose uptake in skeletal muscle cells by inhibiting intracellular insulin signalling through blocking of PI3-K activation.^{46,47} Therefore, it is highly possible that NEFA interfere with insulin-mediated vasodilatation through the same mechanism.

Taken together, the above findings suggest that plasma NEFA elevation for about 2 h in healthy humans results in the reduction of basic vascular NO production and endothelium-dependent vasodilatation, as well as slight increases in BP. Long-term elevations (8 h) on the other hand, additionally blunt insulin-mediated glucose uptake and vasodilatation and produce more pronounced BP increases. It has been postulated that chronic NEFA elevation in insulin-resistant states, apart from interfering with insulin-mediated glucose uptake, is the main factor responsible for the

observed endothelial dysfunction and impaired insulin-mediated vasodilatation (Figure 1).¹⁰ Thus, it can also be one of the basic mechanisms for the increase incidence of hypertension in these patients.

NEFA and oxidant stress

An additional mechanism connecting NEFA and BP elevation could be an increase of oxidative stress.¹⁶ *In vitro* studies support the notion that NEFAs can induce oxidant stress.⁴⁸ *In vivo*, elevation of NEFAs with Intralipid and heparin infusions has been shown to increase the concentrations of various biomarkers of oxidant stress.^{49,50} On the other hand, the contribution of oxidant stress in hypertension development is supported by preliminary animal data,⁵¹ and indirect evidence from studies where diet rich in fruits and vegetables, containing high amounts of antioxidant substances, significantly reduced BP.⁵²

Egan *et al.*¹⁶ suggested that a relatively high intake of antioxidants could be responsible for the limited BP increases in some of the above studies.^{23,38} To test this hypothesis, the investigators administered Intralipid and heparin for 4 h in healthy individuals being for the former 3 weeks on a diet low in antioxidants. This infusion was associated with significant increases in SBP and DBP of 10 and 3 mm Hg, respectively, whereas respective infusion of saline and heparin did not significantly affect BP.²⁵ However, it is obvious that to effectively assess the effect of antioxidants in NEFA-induced BP increase, a comparison of two groups receiving diets of low and high concentrations of antioxidants should be performed.

In a more recent study, these investigators examined basic BP levels and the effects of NEFA elevation in obese hypertensive and lean normotensive subjects under their usual diet, the Dietary Approaches to Stop Hypertension combination diet (DASH-CD), which is rich in antioxidants, and a low-antioxidant diet.²⁷ SBP, DBP and MBP declined significantly in obese hypertensives after 3 and 4 weeks on the DASH-CD compared with values on their usual and the low-antioxidant diet. After 4 weeks on the DASH-CD, BP in obese hypertensives was 8.1/7.4 mm Hg lower than on the low-antioxidant diet. In lean normotensives, SBP tended to rise during the low-antioxidant diet, but the difference compared with that of the DASH-CD was not significant. After 4 h of Intralipid and heparin infusion, SBP increased significantly in obese hypertensives (about 4–5 mm Hg) and in lean normotensives (from 8 to 13 mm Hg) on all three diets and DBP followed similar trends. However, during this acute elevation of FFA, SBP and DBP in both the groups remained lower on the DASH-CD than on the usual and low-antioxidant diets.²⁷ Although these data are intriguing, as direct mechanisms connecting oxidative stress with

hypertension development have not yet been established, future studies should explore this potential pathway linking NEFAs with BP elevation.

Possible trophic actions of NEFAs

Another possible mechanism connecting NEFAs with BP increase is their possible mitogenic actions on the vascular wall, as increased proliferation of vascular smooth muscle cells (VSMCs) has been long connected to vascular hypertrophy and arterial stiffness, one of the basic mechanisms of hypertension development.^{53,54} *In vitro* studies have reported that unsaturated NEFAs, including oleic and linoleic acid, can directly activate the typical and atypical isoforms of protein kinase C (PKC) in various cell types, including VSMCs.^{55–58} Among other deleterious effects on human vasculature, PKC has been shown to play a central role in mitogenic processes in VSMCs.^{59,60} Several studies have clearly shown that NEFAs induce proliferation of VSMCs via PKC activation,^{55–57} implicating a potential involvement of plasma NEFA elevation in vascular remodelling and hypertension in obese subjects. In addition, oleic and linoleic acid were reported to increase the trophic action on VSMC of other growth factors, for example insulin-like growth factor-1 (IGF-1).⁶¹ In the cardiovascular system, IGF-1 is produced from VSMCs after stimulatory effects of various factors, including insulin; thus, its release is also elevated in states of IR and hyperinsulinaemia.⁶²

Other pathways potentially connecting NEFAs with BP elevation

In addition to the mechanisms described so far, preliminary evidence for other actions of NEFAs that can contribute in the development of hypertension also exist (Table 2). For example, NEFAs can counteract with RAAS in several points. In some of the above-mentioned studies, oleic acid did not only induce VSMC proliferation, but also stimulated the mitogenic response of these cells in angiotensin II, findings supporting a synergistic effect of NEFAs and angiotensin II on vascular growth.^{56,57} NEFAs were also reported to activate the expression of angiotensinogen gene in preadipocytes,⁶³ an action possibly contributing in the upregulation of adipose tissue RAAS in subjects with central obesity. Further, an oxidized derivative of linoleic acid was recently reported to stimulate aldosterone production from rat adrenal cells, providing an alternative explanation for unexpectedly elevated aldosterone levels in some obese subjects.⁶⁴

In addition, previous *in vitro* studies suggest that both oleic and linoleic acids are endogenous inhibitors of Na⁺,K⁺-ATPase.^{65,66} Reduction of Na⁺,K⁺-ATPase activity in VSMCs through increase in intracellular Na⁺ and decrease of passive Na⁺–

Table 2 Pathophysiologic mechanisms possibly connecting elevation of NEFA with hypertension

- α_1 -adrenergic activation
- Reduction of NO production from endothelial cells and inhibition of endothelium-dependent vasodilatation
- Inhibition of PI3-K pathway and insulin-mediated vasodilatation
- Increase in oxidant stress
- Induction of VSMC proliferation
- Upregulation of the RAAS system
- Inhibition of Na⁺,K⁺-ATPase in VSMCs

Abbreviations: NEFA, non-esterified fatty acids; NO, nitric oxide; PI3-K, phosphatidylinositol 3-kinase; RAAS, renin-angiotensin-aldosterone; VSMC, vascular smooth muscle cell.

Ca²⁺ exchange or through partial depolarization of cell membrane and activation of voltage-dependent Ca²⁺ channels would result in increased intracellular Ca²⁺ concentration and a relative elevation of vascular tone. Thus, inhibition of Na⁺,K⁺-ATPase has been suggested to promote the development of hypertension.⁶⁷ Reduced activity of Na⁺,K⁺-ATPase has been noted in both animal models and humans with obesity and type II diabetes and has been long implicated in the development of hypertension in these states.^{68,69} Therefore, it can be speculated that NEFA elevation is a basic mechanism for this downregulation of Na⁺,K⁺-ATPase activity.

Conclusions

NEFA elevation has been extensively studied as a cause of IR, β -cell apoptosis, dyslipidaemia, endothelial dysfunction and other disturbances in subjects with obesity and type II diabetes. Less attention has been specifically paid to the possible involvement of NEFAs in the development of hypertension, which is a major contributing factor in increased cardiovascular morbidity and mortality of such subjects. However, several studies on related actions also provide information implicating a connection of NEFAs with BP elevation. A close examination of these data reveals an important acute effect of plasma NEFA elevation on BP as well as several pathways through which NEFA increase can lead to hypertension. Thus, it can be speculated that in subjects with features of the metabolic syndrome chronic NEFA elevation could be an important mechanism contributing to either the development of hypertension in previously normotensives or inadequate BP control in patients with already elevated BP. The above data underline the need for future studies to elucidate this important field.

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EFFECTS OF PRENATAL HYPOXIA ON PULMONARY VASCULAR REACTIVITY IN CHICKENS PRONE TO PULMONARY HYPERTENSION

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Among chickens, meat-producing broiler strains are highly prone to develop severe pulmonary hypertension (PH) that is accompanied by endothelial dysfunction in the conduit extrapulmonary arteries. We hypothesized that exposure to chronic prenatal mild hypoxia would accelerate PH and endothelial dysfunction in smaller intrapulmonary arteries from broiler chickens. Fertilized broiler and layer (White Leghorn, WL) eggs were incubated under normoxic or hypoxic conditions. Endothelium-dependent (tested with acetylcholine, ACh) and -independent (tested with sodium nitroprusside, SNP) relaxations of the caudomedial intrapulmonary artery were studied on fetal day 19 and at 2 weeks post-hatch. The response to acute hypoxia *in vitro* was also studied in the 2 wk-old vessels. Relaxations induced by ACh and SNP were similar in broiler and layer chickens and were unaffected by chronic mild hypoxia during incubation. However, during *in vitro* acute hypoxia the broiler arteries showed a markedly enhanced contraction. Chronic prenatal hypoxia did not affect the response of intrapulmonary arteries to acute hypoxia. We conclude that early endothelial dysfunction is not present in the small pulmonary arteries of fast-growing broilers after incubation under normoxic or hypoxic conditions. The higher susceptibility of the broiler pulmonary arteries to acute hypoxia might, at least partially, explain the higher susceptibility to PH.

Key words: *pulmonary hypertension, endothelium, broiler chicken, hypoxia*

INTRODUCTION

Pulmonary hypertension (PH) is a devastating disease, the pathogenesis of which remains poorly defined (1). Recently, genetic and mechanistic strategies have succeeded in identifying signaling pathways involved in PH (1). The current consensus is that injury to the vascular endothelium and/or smooth muscle cells of the small pulmonary arteries and arterioles may initiate the disease process. The underexpression of vasodilators such as nitric oxide

(NO) or prostacyclin and overexpression of vasoconstrictors such as ET-1 are likely not only to affect pulmonary vasomotor tone but also to promote vascular remodeling (2). However, whether these perturbations represent a cause or consequence of the disease process remains to be elucidated. Animal models in which PH is induced with hypoxia or monocrotaline, have contributed to our understanding of the pathogenesis of the disease (3). Although useful, these models do not completely replicate PH cellular changes and pathophysiology.

Thus, further development of animal models is critical for future PH research.

The chicken (*Gallus gallus*) embryo represents an excellent model for investigating developmental physiology of the cardiovascular system (4, 5). Since the embryo develops outside the mother, effects of external stressors on cardiovascular development can be studied independently of any confounding influences from alterations in maternal hormonal, metabolic, or haemodynamic status. Previous studies of our group have characterized reactivity of isolated pulmonary and systemic arteries from fetal day 15 (of the 21 days of incubation) and evaluated the effects of diverse adverse stimuli, such as hypoxia or malnutrition, on this vascular reactivity (4, 6-13).

Among chickens, broiler strains selected for fast growth and meat yield, are highly susceptible to PH, right-sided congestive heart failure, and ascites (14-18). This pathological aggregate of signs known as PH or ascites syndrome was originally reported to be triggered by environmental factors, such as low temperature or hypobaric hypoxia. However, it later became evident that certain strains of broiler chicken developed PH in the absence of these stimuli (17, 18). In contrast, layer strains, such as the White Leghorn (WL), are highly resistant to severe PH under both normoxic and hypoxic conditions (15). Interestingly, Martinez-Lemus *et al.* demonstrated that acetylcholine (ACh)-induced pulmonary artery endothelial-dependent relaxation was reduced in 1- to 3 wk old broilers when compared with layer chickens (15). In contrast, the responses to ACh were similar in layer and broiler chickens before hatch (19). Therefore, the broiler chicken represents a unique model to obtain information before, during, and after the development of PH in susceptible individuals and to make comparisons with individuals with low propensity for the disease (*i.e.* layer chickens).

In mammals, experimental perinatal exposure to hypoxia increased severity of PH in response to a second hypoxic challenge (20, 21). These data suggest that brief hypoxic exposure during a critical period of lung growth may alter the course of normal pulmonary development, and leaves persistent changes in lung structure and/or function that cause an exaggerated response to adverse stimuli later in life (20). Although mortality from PH in broiler chickens is the highest in weeks five to six, it is thought that the onset occurs much earlier and could even start in the fetal period (17, 18). Some experimental observations indicate that hypoxic conditions already occur in the late fetal phase of broiler chickens susceptible to PH (17, 18). This suggests that prenatal hypoxia may initiate structural changes in the pulmonary system and this may be

decisive in determining the subsequent adaptive incapacity to cope with unfavorable conditions after hatching (18). In the present work, we hypothesized that exposure to chronic prenatal mild hypoxia would induce endothelial dysfunction in pulmonary vessels of broiler chickens and accelerate the development of PH. We therefore analyzed the effects of chronic hypoxia during incubation on the endothelium-dependent and -independent reactivity of intrapulmonary arteries from broiler chickens with high susceptibility to PH. The responsiveness to acute hypoxia was also compared between broiler and layer intrapulmonary arteries.

MATERIAL AND METHODS

Chicken strains

Animal procedures were approved by the Ethical Committee of the K.U. Leuven and were in accordance with the institutional guidelines of the university of Maastricht and the K.U. Leuven. Fertilized eggs from layer (White Leghorn) and broiler chickens were obtained from Breeding Research & Technology Centre van Hendrix Genetics B.V. (Boxmeer, The Netherlands). A pure broiler sire strain, primarily selected for a low feed conversion ratio combined with a high body weight gain and presenting a high susceptibility to PH (based on mortality due to ascites induction at low temperatures) was used. This line was also used by Scheele *et al.* (22) then called 'SS' line. Both broiler and layer chickens were studied at two different developmental stages: fetal day 19 and 2 weeks of age.

Incubation, hypoxia protocol, and posthatch development

All eggs were weighted before start of incubation. Incubation was performed at 37.8°C, 45% humidity and eggs were turned (Incubator model 25HS, Masalles Comercial, Spain) over an angle of 90°/h. Control embryos were incubated under normoxic conditions (21% O₂, 0.03% CO₂). Experimental embryos were incubated under normoxic conditions until day 6 of incubation when eggs were transferred to a second 25HS incubator where hypoxic conditions (15.0 ± 0.3% atmospheric O₂, 0.03% CO₂) were maintained by providing a constant flow of N₂ and compressed air with a flow meter (AGA Gas BV, The Netherlands). The O₂ and CO₂ concentrations in the incubator were monitored with a DrDAQ O₂ sensor (Pico Technology, United Kingdom) and an infrared CO₂ analyzer (Beckman Instruments, Inc., Fullerton, U.S.A.).

Eggs were candled and weighted at day 19 of the 21-d incubation and some fetuses were used for experiments and others allowed to hatch. Eggs used for hatch were placed in individual baskets in a hatcher (PasReform, Zeddam, The Netherlands), maintained at 37.6°C and 50-55% humidity at atmospheric oxygen concentration until hatch. After hatch, body mass was recorded and the chick was individually ring-banded. All chickens were reared in a brooder unit under 23 h/day of continuous light and temperature was decreased (1°C every 48 h) from 35°C at hatch. During the grow-out period chickens were provided *ad libitum* access to water and a standard starter diet containing 2890 kcal of ME/kg and 22.2% CP. All chicks were weighted at post-hatch day 7 and at day 14.

Recording of arterial reactivity

The 19-day fetus and the 2-wk-old chickens were euthanized by decapitation, and the heart and lungs were removed, weighed, and immersed in ice-cold Krebs-Ringer bicarbonate (KRB) solution (in mmol/L: NaCl 118.5, MgSO₄ * 7 H₂O 1.2, KH₂PO₄ 1.2, NaHCO₃ 25.0, KCl 4.7, CaCl 2.5 and glucose monohydrate 5.5). With the aid of a dissecting microscope, the caudomedial intrapulmonary artery (23) was carefully dissected free of surrounding tissue and cut into rings with a length of 1.7-2 mm (embryos) or 3-4 mm (2-wk-old chickens). The isolated fetal arteries were mounted between an isometric force transducer (Kistler Morce DSC 6, Seattle, WA) and a displacement device in a myograph (model 610M, Danish Myotechnology, Aarhus, Denmark) using two stainless steel wires (diameter 40 µm). The arteries from the 2-wk-old chickens were mounted between two hooks in a 5 mL organ bath. One hook was attached to the chamber and the other to an isometric force transducer (model PRE 206-4, Cibertec, Madrid, Spain). The isometric force signal was amplified, A/D converted (PowerLab, AD-Instruments, Castle Hill, Australia) and recorded (Chart v3.4, AD-Instruments, Castle Hill, Australia), as previously described (4, 7-12, 14, 15, 24). During mounting and experimentation, the KRB buffer was maintained at 39°C and aerated with 95% O₂-5% CO₂ (fetal arteries) or 21% O₂- 5% CO₂- 74% N₂ (2-wk-old chicken arteries). Pilot experiments demonstrated that bubbling the organ chamber with 95% or 21% oxygen did not affect the contractions induced by KCl or the relaxations induced by acetylcholine (ACh) or sodium nitroprusside (SNP). Embryonic arteries were stretched to their individual optimal lumen diameter, *i.e.*, the diameter at which they developed the strongest contractile response to 62.5

mmol/L K⁺, using a diameter-tension protocol as previously described (4, 8, 9, 24). The arteries from 2-wk old animals were stretched to a resting tension of 9.8 mN, which was found to be the optimal tension for these preparations in pilot experiments. The vessels were allowed to stabilize for 30 minutes before the start of each experiment. Relaxing agonists were evaluated during contraction induced by 62.5 mmol/L K⁺. Concentration-response curves for ACh (10⁻⁹-10⁻⁴mol/L), and the nitric oxide (NO) donor SNP (10⁻⁸M-10⁻⁴mol/L) were constructed. Some experiments were performed in the presence of the NO synthase inhibitor N^ω-Nitro-L-arginine methyl ester (L-NAME, 10⁻⁴mol/L).

The response of the pulmonary arteries to acute hypoxia was assessed in a separate group of experiments. The organ chambers were covered with plastic foil and an acute decrease in O₂ tension was induced by switching the bubbling gas mixture from 21% O₂-74% N₂-5% CO₂ (pO₂ 17 ± 1 kPa) to 95% N₂-5% CO₂ (pO₂ 2.3 ± 0.4 kPa) (5, 25). Each vessel was exposed to two consecutive hypoxic challenges. After the first exposure, the organ chambers were reoxygenated and the tissues were allowed to recover for 20-30 minutes. During the second hypoxic challenge the vessels were consecutively exposed to ACh (10⁻⁶M), SNP (10⁻⁴M) and papaverin (10⁻⁴M).

Assessment of right cardiac hypertrophy

To evaluate right cardiac hypertrophy, the right ventricular index (RVI) was determined. From harvested hearts, the atria were removed, and the ventricles were separated. Ventricles were weighted, and RVI was calculated as the ratio of right ventricle (RV) to left ventricle plus septum (LV + S) mass. The small size of the hearts of the 19-day embryos precluded a proper separation of the right ventricle. These hearts were fixed in 10% phosphate-buffered formalin for 24 h and thereafter kept in 70% ethanol. Then, hearts were paraffin embedded, and three coronal 4-µm sections, at the level of aorta lumen, were stained with von Gieson's stain. Slices were digitally recorded and analyzed by a computerized morphometry system (Quantimet 570, Leica) to determine RV area. RVI was calculated as the ratio of RV to LV + S area, as previously described (8).

Venous blood gases

Using a fine needle, a sample of blood (approximately 200 µL) from the wing vein or jugular vein of 2-wk-old chickens was drawn into a heparinized syringe and immediately analyzed with a GEM Premier 3000 blood gas analyzer

(Instrumentation Laboratory, Lexington, U.S.A.) to determine values of pH, pCO₂ and pO₂.

Data analysis

Results are shown as means (SD) of measurements in *n* animals. For clarity, results in the figures are shown as means \pm SE. Contractions are expressed in terms of active wall tension (N/m), calculated as the force divided by twice the length of the arterial segment, while the relaxant responses are expressed as the percentage of reduction of the contraction induced by K⁺. Sensitivity (expressed as pD₂ = log EC₅₀) and maximal relaxation (E_{max}) to agonists was determined for each artery by fitting individual concentration-response data to a non-linear sigmoidal regression curve and interpolating (Graphpad Prism version 2.01; GraphPad Software Inc, San Diego, CA, U.S.A.). Differences between mean values were assessed by one-way ANOVA followed by the Bonferroni post hoc t-test. Differences in the percentage of hatching were assessed by the Fisher's test. Differences were considered significant at a *p* < 0.05. All analyses were performed using a commercially available statistics package (GraphPad InStat version 3.00, GraphPad Software Inc, San Diego, CA, U.S.A.).

RESULTS

Effects of hypoxia on embryonic and post-hatch growth

Either prior to and after 19 days of incubation, WL eggs were lighter than broiler eggs (*Table 1*). Incubation under normoxic or hypoxic conditions induced a similar reduction in egg masses. The percentage of hatching of fertile eggs was significantly lower in the normoxic broiler (78,00%, *n*=50) than in the normoxic WL group (96,97%,

n=33, *p*=0.0231). Hypoxic incubation induced a further significant decrease (*p*=0.0001) in the percentage of hatching success in the broiler (30.10%, *n*=103) but not in the WL chickens (87.88% *n*=33).

Fetal and post-hatch body masses are shown in *Table 1*. At fetal day 19, broilers showed higher body masses than WL chickens (*p*<0.0001). At hatch, one week and two weeks of age, normoxic broilers also displayed higher body masses than normoxic WL chickens (*p*<0.001 at all ages). Chronic hypoxia induced a significant growth restriction in broilers and layers at fetal day 19. However, at hatch (after two days of normoxic incubation) normoxic and hypoxic chicks showed no significant differences in body masses. Interestingly, growth differences were again present between normoxic and hypoxic broilers at the ages of one and two weeks. In contrast, hypoxic leghorns did not show significant differences in body mass at the ages of one and two weeks when compared to normoxic leghorns.

Contraction to KCl

Intrapulmonary arteries isolated from 19-d fetuses and 2-wk-old chickens responded to depolarizing high- K⁺ solution with a tonic contraction. At fetal day 19 contractions to KCl were similar in broiler (0.30 SD 0.16, *n*=23) and WL intrapulmonary arteries (0.32 N/m, SD 0.17, *n*=10). When compared with the fetal arteries, the 2 wk-old pulmonary arteries showed significantly higher KCl-induced contractions (broiler: 0.85 N/m, SD 0.49, *n*=25, *p*<0.01 vs fetal broiler; WL: 0.71 N/m, SD 0.41, *n*=17, *p*<0.05 vs. fetal WL) but no significant differences between the two chicken strains were observed. Chronic prenatal hypoxia did not affect the responses to KCl of the arteries from the 19-d broiler fetus (0.37 N/m, SD 0.12, *n*=8), the 2-wk-old broiler (0.86 N/m, SD 0.58, *n*=17) or the 2 wk-old WL (0.67

Table 1. Masses (g) of the eggs (at day 0 and 19 of incubation), fetuses (day 19), and chickens (at hatching and at 1 and 2 weeks post hatch) of the broiler and White Leghorn (WL) strains incubated from day 6 to 19 under normoxic or hypoxic conditions. **p* < 0.05 for differences between broiler and WL within the same experimental group (fetal normoxia/hypoxia). #*p* < 0.05 for differences between normoxia and hypoxia within the same chicken strain. Results are presented as mean (SD) of *n* eggs/animals.

	Egg d0	Egg d19	d19 fetus	hatch	1 wk	2 wk
Broiler normoxia	64.58 (6.55)* ³⁹	57.29 (6.15)* ³⁹	31.43 (0.63)* ^{#15}	47.25 (5.46)* ³⁹	159.03 (26.37)* ^{#39}	403.22 (90.55)* ^{#37}
Broiler hypoxia	62.22 (5.85)* ³³	55.25 (5.70)* ³²	25.55 (0.47) ¹²	45.91 (5.36) ³¹	131.93 (19.61)* ³¹	330.52 (54.96)* ³¹
WL normoxia	60.22 (3.51) ³²	53.30 (3.67) ³²	29.17 (0.25) ^{#95}	42.46 (2.81) ³²	61.46 (9.80) ²⁸	107.21 (14.36) ²⁸
WL hypoxia	59.11 (4.08) ²⁹	52.11 (3.84) ²⁹	25.89 (0.37) ⁴⁹	41.41 (2.68) ²⁹	56.32 (5.42) ²⁵	99.77 (13.23) ²²

N/m, SD 0.50, $n=22$). Contractions to KCl were not significantly affected by the presence of L-NAME (data not shown).

Relaxation responses to ACh and SNP

Relaxing responses were studied during KCl-induced contraction. No differences between the chicken lines were observed at any age in the potency and efficacy of ACh and SNP (Table 2, Fig. 1). No developmental changes were observed in the responses to ACh or SNP when fetal and 2-wk-old arteries were compared. The NOS inhibitor L-NAME significantly impaired ACh-induced relaxation, but in its presence no age- or strain-related differences in the effects of ACh were observed (data not shown). As shown in Fig. 1 and Table 2, chronic hypoxic incubation did not induce

significant alterations in the pulmonary artery relaxations elicited by ACh and SNP from broiler (studied at fetal day 19 and 2 wk of age) and WL chickens (studied only at 2 wk of age). L-NAME significantly reduced the sensitivities to ACh in pulmonary arteries from the hypoxic chickens but ACh-induced relaxation in the presence of L-NAME was not significantly different between hypoxic and normoxic animals (data not shown).

Hypoxic vasoconstriction

The intrapulmonary arteries of 2-wk-old broiler and WL chickens responded to acute hypoxia (applied on top of resting tension) with a tri-phasic (*i.e.* contraction-relaxation-contraction) (Fig. 2A) or a monophasic contraction. Hypoxia-induced contraction reached a plateau after 10-15 minutes

Table 2. Maximal relaxation (E_{max}) and sensitivities (pD_2) to acetylcholine (ACh), and the NO donor sodium nitroprusside (SNP) in intrapulmonary arteries from 19-day fetal and 2-wk-old broiler and White Leghorn (WL) chickens incubated under normoxic or hypoxic conditions from day 6 to 19. Results are presented as mean (SD) of n animals.

		d19 fetus			2-wk chicken			
		Broiler normoxia	Broiler hypoxia	WL normoxia	Broiler normoxia	Broiler hypoxia	WL normoxia	WL hypoxia
ACh	E_{max}	91.59 (8.42) 7	89.46 (7.62) 8	88.21 (6.89) 11	82.36 (6.70) 8	82.36 (6.70) 8	71.42 (10.36) 5	90.47 (16.99) 10
	pD_2	7.19 (0.49)	7.03 (0.17)	6.96 (0.34)	7.18 (0.34)	7.17 (0.34)	6.99 (0.37)	7.11 (0.37)
SNP	E_{max}	92.47 (11.74) 7	81.30 (4.79) 8	91.34 (4.98) 10	85.38 (9.02) 7	92.15 (9.11) 8	81.05 (24.03) 5	96.88 (14.68) 9
	pD_2	6.77 (0.70)	6.33 (0.11)	6.88 (0.80)	6.46 (0.25)	6.71 (0.34)	6.41 (0.65)	6.67 (0.48)

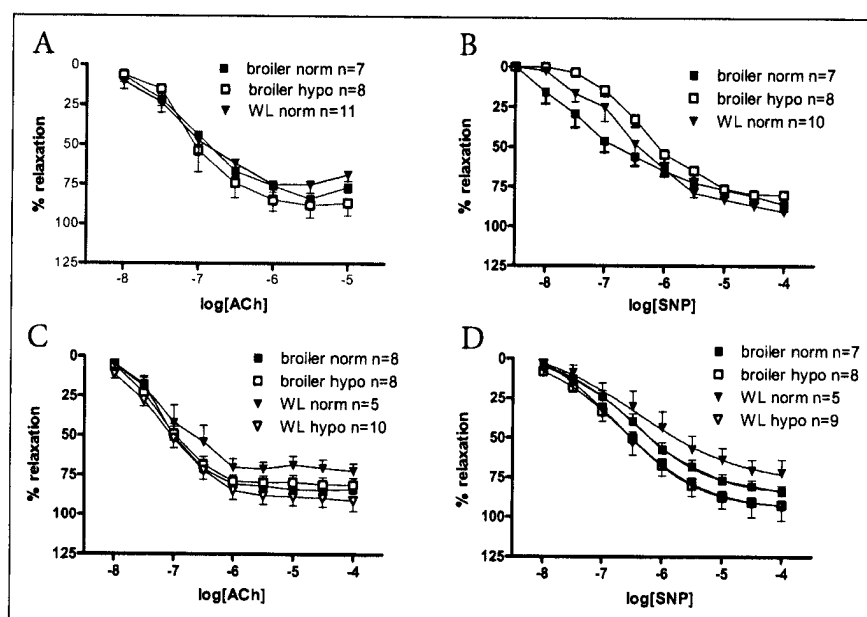


Fig. 1. Concentration-dependent relaxing effects of acetylcholine (ACh, A and C) and sodium nitroprusside (SNP, B and D) in intrapulmonary arteries from 19-d fetal (A and B) and 2-wk old (C and D) broiler and White Leghorn (WL) chickens. The eggs were incubated under normoxic or hypoxic (15% O_2 from day 6 to 19) conditions. Intrapulmonary arteries were contracted with KCl (62.5 mM) and relaxations are expressed as percentage of reduction of KCl-induced contraction. Each point represents the mean \pm SE of n animals.

and was completely reversed upon reoxygenation with 21% O₂. A second hypoxic challenge induced a contraction that tended to be larger. The area under the curve (normalized to the KCl-induced contraction in each individual vessel) of the two consecutive hypoxic challenges was significantly higher in the broiler than in the WL arteries (*Fig. 2B*). When consecutively applied during the second hypoxic challenge, ACh (1 μ M) and SNP (10 μ M) induced a ~25% and a ~95% relaxation of the tone

evoked by hypoxia, respectively (*Fig. 2C and D*). When added after SNP, papaverine induced only a small further relaxation (*Fig. 2A*). The relaxations induced by ACh, SNP, and papaverine in hypoxia-contracted arteries were similar in the broiler and the WL arteries (*Fig. 2D*). Chronic fetal hypoxia did not significantly modify the contraction induced by acute hypoxia or the relaxations of the hypoxic contractions evoked by ACh, SNP, or papaverine in 2 wk chickens (*Fig. 2D*).

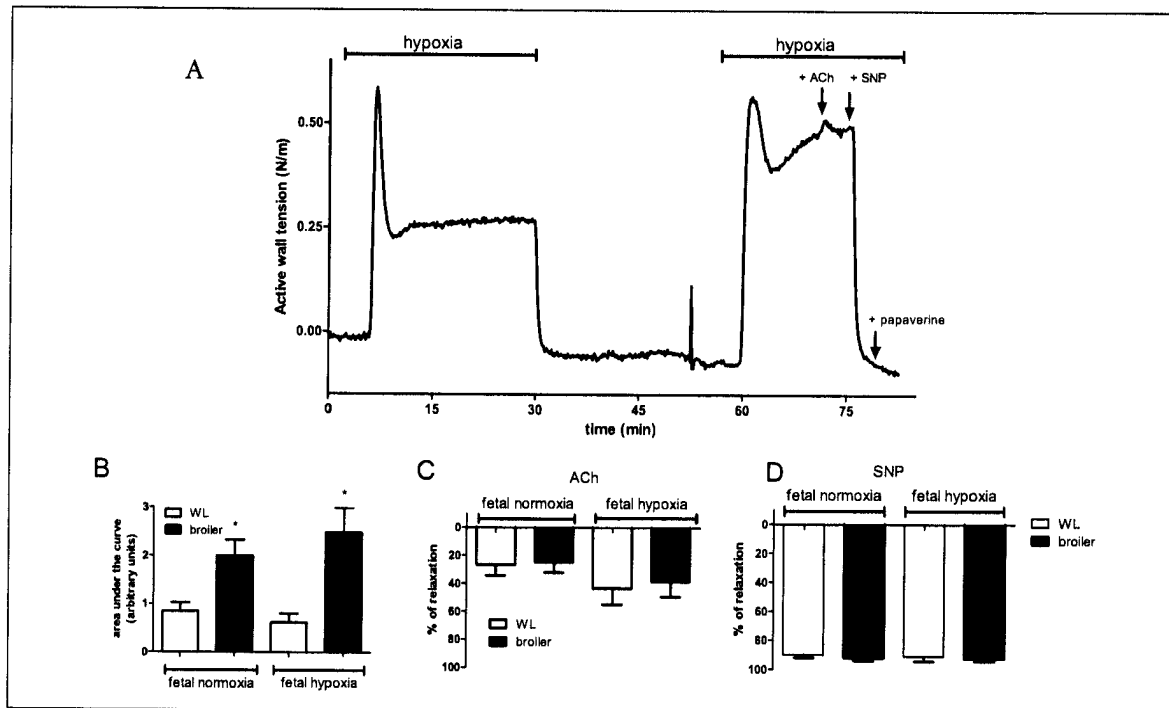


Fig. 2. Hypoxic vasoconstriction of intrapulmonary arteries from 2-wk-old broiler and White Leghorn (WL) chickens. **A:** Representative tracing showing the effects of two consecutive hypoxic challenges (switching the gas mixture in the organ chamber from 21% O₂-74% N₂-5% CO₂ to 95% N₂-5% CO₂) in a broiler vessel. Hypoxia induced a tri-phasic (*i.e.* contraction-relaxation-contraction) or a monophasic contraction. Hypoxia-induced contraction reached a plateau after 10-15 minutes and was completely reversed by switching the gas mixture to 21% O₂. A second hypoxic challenge induced a contraction that tended to be larger and also reached a plateau after 10-15 minutes. When consecutively applied during the second hypoxic challenge, acetylcholine (ACh, 1 μ M) and SNP (SNP, 10 μ M) induced a ~25% and a ~95% relaxation of the tone evoked by hypoxia. Further addition of papaverine (0.1 mM) induced a minimal additional relaxation. **B:** Area under the curve (corrected for KCl-induced contraction) of the contraction induced by 30 min. of acute hypoxia (two consecutive challenges of 15 min) in chicken intrapulmonary arteries. * $p < 0.05$ for comparison broiler vs. WL within the same experimental group (fetal normoxia/hypoxia). **C and D:** Summary of the relaxations induced by ACh and SNP in acute hypoxia-contracted arteries. Each bar represents the mean \pm SE. the n of the different groups were $n = 12$ for WL normoxia, $n = 12$ for WL exposed to fetal hypoxia, $n = 17$ for broiler normoxia and $n = 9$ for broilers exposed to fetal hypoxia.

Table 3. Venous blood pH, pCO₂ and pO₂ values of 2-wk-old broiler and White Leghorn (WL) chickens incubated under normoxic or hypoxic conditions. * $p < 0.05$ for differences between broiler and WL within the same experimental group (fetal normoxia/hypoxia). # $p < 0.05$ for differences between normoxia and hypoxia within the same chicken strain. Results are presented as mean (SD) of n animals.

	pH	pCO ₂ (mmHg)	pO ₂ (mmHg)
Broiler normoxia (n=28)	7.35 (0.07)*#	42.89 (5.85)*	42.57 (10.20)*
Broiler hypoxia (n=34)		42.44 (5.84)*	41.06 (8.71)*
WL normoxia (n=34)	7.41 (0.06)	33.79 (6.29)	52.41 (7.30)
WL hypoxia (n=31)	7.42 (0.06)	32.32 (5.26)	52.81 (6.46)

Assessment of right ventricle hypertrophy

The RVI (RV area/LV + septum area) of the hypoxic 19-d broiler fetuses (15.31, SD1.59, n=11) was significantly higher than the RVI of the normoxic broiler fetuses (13.20 SD2.20, n=10, $p<0.024$). At the age of 2 weeks, WL chickens showed a slightly larger (0.37g SD0.05, n=18) but significant RVI (RV mass/LV + septum mass) than broiler chickens (0.32g, SD0.04, n=22, $p<0.05$). Chronic hypoxia did not significantly affect the RVI of the 2-wk-old broiler (0.33g, SD0.07, n=29) or WL chickens (0.34g, SD0.05, n=19).

Blood gasses

The values of pH, pCO₂ and pO₂ were determined in venous blood samples from 2-wk-old chickens. The results are summarized in Table 3. Normoxic broiler chickens showed significantly lower pH ($p<0.01$) and pO₂ values ($p<0.001$) and significantly higher pCO₂ values ($p<0.001$) than normoxic layer chickens. Chronic fetal hypoxia did not lead to different pO₂ or pCO₂ values than the respective normoxic controls of the same strain. However, the pH of hypoxic broilers was higher than the pH of normoxic broilers.

DISCUSSION

In the present work, we analyzed endothelium-dependent and -independent relaxations and responsiveness to acute hypoxia of intrapulmonary arteries from broiler and layer chickens and we evaluated the effects of chronic prenatal mild hypoxia on these vascular responses. We observed that pulmonary artery endothelium-dependent relaxation was similar in broiler and layer 19-day embryos and 2-wk-old chickens and was unaffected by chronic fetal hypoxia. However, we found an interesting difference between broiler and layer (WL) intrapulmonary arteries: hypoxic pulmonary vasoconstriction (HPV) was markedly enhanced in the broiler vessels.

Similar acetylcholine-induced relaxation in intrapulmonary arteries from broiler and layer chickens

Several experimental data suggest that reduction of endothelial NO synthesis is associated with increased pulmonary vasomotor tone and vascular remodeling in pulmonary hypertensive broilers (26, 27). Martinez-Lemus *et al.* (15, 19) reported that ACh-induced endothelium-dependent relaxation is

impaired in the extrapulmonary arteries of 2-wk-old broilers when compared with those of layer chickens. This impairment of ACh-induced relaxation was not present when the vessels from pre-hatched broiler and WL chickens were analyzed (19). Similarly, we have not observed strain-related differences in the relaxations induced by ACh and the NO donor SNP in 19-d fetal intrapulmonary arteries. However, the findings of Martinez-Lemus *et al.* (15, 19) in 2-wk-old extrapulmonary arteries are not confirmed in the present work using smaller vessels. In line with our results, Oka (28) observed, in a rat model of PH, a marked decrease in both ACh- and SNP-induced relaxations in large but not in small pulmonary arteries. It is well known that the various segments of pulmonary circulation are characterized by a marked heterogeneity in structure and function (29, 30). In the chicken embryo, the extrapulmonary preductal segment of the pulmonary artery displays the morphology of an elastic artery, similar to that of aorta, until it bifurcates into two typically muscular arteries, the ductus arteriosus and the post-ductal intrapulmonary pulmonary arteries (12, 31). Moreover, the preductal and the postductal segment of the pulmonary artery do not share a common embryonic origin (31). However, and although in our experiments we used smaller vessels than the ones investigated by Martinez-Lemus *et al.* (15, 19), they were still relatively large arteries whose reactivity should be cautiously extrapolated to resistance vessels and to *in vivo* situation.

Besides the use of different segments of the pulmonary circulation, a second major difference between our experimental design and the one of Martinez-Lemus *et al.* (15, 19) is the use of KCl instead of endothelin-1 to contract the vessels. Among others, endothelium-dependent relaxation is achieved by combined vasodilator effects of endothelium-derived prostacyclin, NO, carbon monoxide, and endothelium-derived hyperpolarizing factor (EDHF) (32). Contribution of these factors to relaxation varies across species, vascular beds, and also with the agents used to stimulate the endothelium and the vascular smooth muscle (32). We studied ACh-induced relaxation during contraction of the pulmonary arteries with high K⁺ depolarizing solution that precludes the action of relaxing agents, such as EDHF, which act through hyperpolarization of vascular smooth muscle cells. Moreover, high K⁺ solution also induces depolarization of the endothelial cells producing an impairment of endothelial function (33). Thus, the use of ET-1 as precontractile agent might have been more adequate for the study of ACh-induced relaxation in our experiments. However, and although ET-1 contracts chicken intrapulmonary

arteries (8, 9), it produces, particularly in the 2-week-old chickens, marked superimposed rhythmic contractions (with an amplitude up to 150% of the contraction induced by 62.5mM K⁺), which precluded the use of ET-1 as a pre-contractile agent in our experiments.

Chronic in ovo hypoxia did not affect endothelium dependent-relaxation of chicken intrapulmonary arteries

We hypothesized that exposure to chronic prenatal mild hypoxia would induce endothelial dysfunction in pulmonary arteries of broiler chickens and accelerate the development of PH. However, we observed that ACh-induced relaxation of the broiler intrapulmonary arteries was not affected by chronic fetal hypoxia. Moreover, broilers incubated under hypoxic conditions did not show right ventricle hypertrophy at 2 weeks of age, suggesting a lack of effect of hypoxic incubation on the development of PH at least at this age. Accordingly, Hassanzadeh *et al.* (34) demonstrated that broiler chickens that were incubated and hatched at high altitude but reared at low altitude showed a similar incidence of PH than broilers incubated, hatched and reared at low altitude. Interestingly, they also reported that incidence of PH was lower in broilers incubated and reared at high altitude than in broilers incubated at low altitude and reared at high altitude. This suggests that in ovo hypoxia protected against the effects of post-hatching hypoxia or, alternatively, selected individuals with a lower susceptibility to hypoxia. In our experiments a similar process of selection might have occurred because hypoxia during incubation produced a marked decrease in the hatchability of broiler chickens. On the basis of our results, we can only speculate on the primary mechanisms mediating the differential susceptibility of broiler chickens to hypoxia. Hypoxia-inducible factor 1 (HIF-1) is a master regulator of oxygen homeostasis that controls transcriptional responses to hypoxia (35). As in mammals, HIF-1 plays major roles in the chicken cardiovascular system development (36, 37) and in the pathophysiology of conditions that involve tissue hypoxia (38). Therefore, HIF-1 and its target genes appear as excellent candidates for future studies focused on determining the transcription factors that regulate the effects of pre- and postnatal hypoxia in the broiler chicken.

Hypoxic pulmonary vasoconstriction is enhanced in broiler intrapulmonary arteries

Hypoxic pulmonary vasoconstriction (HPV) is a highly conserved adaptive physiological mechanism

that optimizes oxygen saturation of pulmonary venous blood by increasing pulmonary vascular resistance in poorly aerated regions of the lung (39, 40). Isolated pulmonary arteries have been used by numerous investigators for the study of the physiological properties, pharmacological influences and nature of the mechanism of HPV (41). Among these studies, there are marked differences in the order of the pulmonary arteries studied and in the utilization of a vasoconstrictor to generate an active tension (termed "pre-contraction") before eliciting HPV. The confounding effects of the pre-contraction are unknown, but HPV in vivo does not require it, nor is pre-contraction required for robust HPV in small intrapulmonary artery rings (30). Very recently, Russel *et al.* (40) demonstrated HPV in chicken extrapulmonary arteries pre-contracted with KCl, whereas Odom *et al.* (42) reported, in the same vessel, no response to hypoxia while at resting tension. In the present work, we observed a consistent and reproducible constrictor response to hypoxia in 2-wk-old chicken intrapulmonary arteries at resting tension. Interestingly, we found that arteries isolated from broiler chickens showed greater constrictor responses to acute hypoxia than those from WL chickens. The enhanced contractile responses of the broiler arteries were relatively specific for hypoxia because K⁺-induced contraction was not significantly different between the two chicken lines. Taken together our data suggest that the increased susceptibility of broiler chickens to hypoxia-induced PH is, at least partly, an intrinsic property of pulmonary arteries independent of the effects of circulating vasoactive mediators or other extravascular signaling mechanisms.

Differential pulmonary artery responses to hypoxia between strains of the same animal species have been previously reported (43, 44). The Madison strain of Sprague-Dawley rat has greater pulmonary vasoresponsiveness to acute hypoxia than the Hilltop. In contrast, the Hilltop strain has an attenuated pulmonary hypertensive response to chronic hypoxia (43, 44). A difference between the two strains in pulmonary artery endothelial function, and more particularly with respect to EDHF, appears to contribute to the differences in the susceptibility to HPV and PH (44). On the other hand, fawn hooded rats, a strain in which PA occurs spontaneously, have reduced vasoconstriction to acute hypoxia when compared with Sprague-Dawley rats (45). This suppression of acute HPV has been proposed to be the result of a disruption of the mitochondrial oxygen sensing mechanisms in the pulmonary arteries (45). The mechanisms responsible for the enhanced HPV in the broiler chicken remain to be investigated. However, the fact that ACh- and SNP-induced

relaxations of the hypoxia-constricted pulmonary arteries were similar in the broiler and in the WL suggests a lack of involvement of the NO/cGMP pathway in the differences in responsiveness to hypoxia between the vessels. In contrast to our results, Odom *et al.* (42) reported that hypoxia reduced ACh-induced relaxation in isolated extrapulmonary arteries from broilers but had no significant effect on those from layer chickens. Again, the differences concerning the size of the arteries studied by us and by Odom *et al.* (42) may explain the discrepancies between results.

Chronic fetal mild hypoxia did not alter the response of the 2-wk-old pulmonary arteries to acute hypoxia. Previous studies in mammals have reported conflicting results with respect to the vasoconstrictor response to an acute hypoxic challenge after chronic hypoxia and reduced, enhanced, or unchanged HPV have been reported after exposure to chronic alveolar hypoxia (46). In humans, exposure to transient hypoxic pulmonary hypertension in the first days of postnatal life was related to an enhancement of HPV in adult life (47). However, and to the best of our knowledge, the effects of prenatal hypoxia on HPV have not been analyzed in any animal model.

Effects of chronic hypoxia on embryonic and post-hatch growth

In the avian embryo, hypoxia during incubation consistently decreases body growth (8, 11, 48). However, when eggs are transferred to normoxic conditions for the last two days of incubation a catch up is produced and the hatching weight is similar to that of normoxic-incubated chickens (8, 10). The chick embryo consumes 60% more oxygen between the start of pulmonary breathing and hatching compared to earlier stages (49). Exposure to hypoxia during that period appears to be a critical limiting factor for the rapid growth that occurs during the last days of incubation. In the present study, we confirmed those findings. Furthermore, we found that, although the body mass at hatch was not reduced, broilers exposed to chronic in ovo hypoxia showed lower mean body masses compared to normoxic broilers at both 1 and 2 wk of age. This is in accordance with Hassanzadeh *et al.* (34) who found that broilers incubated and hatched at high altitude (2000 m) and reared at sea level showed (at hatching and 14 days of age) lower mean body masses than broilers incubated at sea level. It is generally accepted that slowing of the initial rapid growth during the first weeks of broiler raising, may have a beneficial effect on PH incidence (50). Interestingly, in humans, the fetal metabolic and

hormonal responses to intrauterine growth restriction and to rapid postnatal catch up growth are likely to be key to the early pathogenesis of adulthood cardiovascular and metabolic diseases (51).

Venous blood gases

In comparison to 2-wk-old layers, age-matched broilers had a lower pH and pO₂ and higher pCO₂ in their venous blood. However, fetal hypoxic chickens displayed, at 2 weeks of age, similar venous pO₂ and pCO₂ than normoxic strain-matched chickens. The cumulative effects of high metabolic oxygen demand, incapacity to increase cardiac output in the face of hypoxemia, and elevated intrapulmonary shunt fraction are proposed as the main factors determining the development of hypoxemia in fast-growing broilers (52). On the other hand, chronic hypercapnia in the broiler chicken has been explained as the result of venous blood containing excess CO₂ entering the arterial circulation through an increased number of intrapulmonary shunts (52). Several investigators (53-55) demonstrated that broilers with PH had a higher venous pCO₂ at 6-7 weeks than PH-resistant broilers and it was suggested that the elevated pCO₂ may be a contributing factor to broiler PH (52). Moreover, a high venous pCO₂ tension measured at post-hatch day 11 was a reliable predictor for PH at 5 weeks of age (55). Chronic hypercapnia is also a common finding in human patients with progressive hypoxic lung disease, and it is a common clinical observation that significant PH does not develop in the presence of hypoxic lung disease unless hypercapnia is also present (56). However, the association between elevated arterial pCO₂ and increased PA pressure may simply reflect the presence of more severe lung disease in hypercapnic individuals and experimental evidence indicates that the augmentation of PH observed in the presence of CO₂ retention is not caused by a hypercapnia-induced elevation in pulmonary vascular resistance or hypoxic responsiveness (57). In fact, chronic hypercapnia inhibited the development of hypoxic pulmonary vascular remodeling and RV hypertrophy, attenuated the polycythemic response to hypoxia, inhibited the pulmonary vasoconstrictor response to acute hypoxia and protected against the impairment of endothelium-dependent relaxation caused by chronic hypoxia in a rat model of PH (57). Hypercapnia was demonstrated to have potent antioxidant effects and it was suggested to protect against hypoxic pulmonary hypertension through attenuation of hypoxia-induced oxidant stress in the lung (58). Interestingly, broiler chickens incubated under increased concentrations of CO₂ showed a lower incidence of PH during the growing period (54).

Concluding remarks and perspectives

Avian models are used to study atherosclerosis, hypertension, hypercholesterolemia, or teratogenic conditions (59). The broiler appears to be an interesting alternative model for the understanding of the genetics (60) and the developmental pathophysiology of PH and for the investigation of prenatal insults such as chronic hypoxia or hormonal alterations (61) on the susceptibility to postnatal PH. However, from our data it is concluded that an early endothelial dysfunction is not present in the small pulmonary arteries of fast-growing broilers after fetal exposure to normoxic or hypoxic conditions. In contrast, the *in vitro* response to acute hypoxia of the broiler pulmonary arteries was markedly enhanced. The most widely accepted theory to explain HPV proposes that it results from the coordinated actions of a sensor (proximal mitochondrial electron transport chain) which generates a diffusible mediator (a reactive O₂ species) that regulates effector proteins (voltage-gated K⁺ channels) (39). Whether the differences in hypoxic pulmonary vasoconstriction between WL and broiler arteries is related to differences in the sensor, the mediator or the effector warrants further investigation.

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